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Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition

This document provides performance guidelines for the immunophenotypic analysis of neoplastic hematolymphoid cells using immunofluorescence-based flow cytometry; for sample and instrument quality control; and precautions for acquisition of data from neoplastic hematolymphoid cells.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Abstract

The importance of immunophenotyping for the proper diagnosis and management of patients with hematolymphoid neoplasia necessitates the development of guidelines for the appropriate performance of these techniques in the clinical laboratory. CLSI document H43-A2—*Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition* addresses issues of safety, specimen collection and transportation, sample preparation, immunofluorescent staining, instrument quality control, data acquisition, and data storage for the application of flow cytometry to the immunophenotypic analysis of these disorders. This document builds on CLSI document H42—*Enumeration of Immunologically Defined Cell Populations by Flow Cytometry*.

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Foreword

H43-A2 establishes performance guidelines for the immunophenotyping of specimens potentially harboring neoplastic hematolymphoid cells by flow cytometry. It is designed to help clinical laboratories using different commercially available instruments and reagents obtain comparable results, and to aid these laboratories in the development of quality assurance procedures that are specifically applicable to such cases.

This document follows a related document, H42—*Enumeration of Immunologically Defined Cell Populations by Flow Cytometry*. In some respects, some sections of the current document—particularly those that cover specimen collection and transportation, safety, instrument quality control (QC), and data storage—are similar to those in H42. However, issues specific to the study of samples of leukemia and lymphoma are covered in sections on sample preparation, sample staining and QC procedures, data acquisition, data analysis, and result reporting and interpretation.

There has been a substantial expansion of the application of flow cytometry (FCM) in hematolymphoid neoplasia since the previous publication of this approved guideline as H43-A in 1998. Instrumentation has improved, routine use of four or more color FCM has expanded, and new applications in assessment of hematolymphoid malignancies are under constant development. Current classification systems rely upon immunophenotype for diagnosis, increasing the importance of clinical FCM in analysis of hematolymphoid neoplasia. In addition, the clinical utility of flow cytometric analysis in new disease categories, such as myelodysplastic syndrome, was established and prognostic immunophenotypic markers have been described. Flow cytometric immunophenotyping also plays a vital role in evaluation of patients for monoclonal targeted therapies. The document has therefore been revised to reflect these advances.

A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, ISO (International Organization for Standardization), and CEN (European Committee for Standardization) documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, CLSI recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In order to align the usage of terminology in this document with that of ISO, the term *accuracy,* in its metrological sense, refers to the closeness of the agreement between the result of a (single) measurement and a true value of a measurand, and comprises both random and systematic effects.

The term *diagnostic sensitivity* is combined with the term *clinical sensitivity,* and correspondingly the term *diagnostic specificity* is combined with the term *clinical specificity*, because in Europe, the term "clinical" often refers to clinical studies of drugs under stringent conditions.

All terms and definitions will be reviewed again for consistency with international use, and revised appropriately during the next scheduled revision of this document.

Key Words

Acute leukemia, autofluorescence, B-cell, chronic leukemia, cluster differentiation system, color compensation, erythrocyte lysing, flow cytometry, fluorescence intensity, fluorochrome, forward angle light scatter (FSC), gate, hematolymphoid neoplasia, hematopathology, histogram, immunophenotyping, list mode, lymphoma, monoclonal antibody, multiparameter display, myelodysplastic syndrome, NK-cell, procedural control, quality control, side scatter (SSC), T-cell, tandem conjugate, viability

Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition

1 Scope

This document establishes performance guidelines for the flow cytometric immunophenotypic analysis of samples from patients with known or suspected hematolymphoid neoplasia. The World Health Organization (WHO) classification system relies upon morphology, clinical history, immunophenotype, and cytogenetics for diagnosis of hematolymphoid neoplasia. Therefore, immunophenotypic analysis of hematolymphoid neoplasia is crucial for the accurate diagnosis and classification of these complex malignancies. It is not within the scope of this document to recapitulate the criteria used to diagnose leukemias and lymphomas. Readers are referred to *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues* (Jaffe ES, Harris N, Stein H, Vardiman JW, eds. Lyon, France: IARC Press; 2001). Because this document is intended primarily for laboratory workers in FCM, it cannot describe all the possible clinical situations in which flow cytometric analysis of leukemia or lymphoma is or is not appropriate.

This document includes guidelines for phenotyping cases of acute and chronic leukemias, non-Hodgkin's lymphomas, plasma cell neoplasms, and myelodysplastic syndromes. The subcommittee recognizes that most of the principles used to approach chronic lymphoid leukemias can also be applied to the study of lymphomas, so these problems are considered together. Special problems unique to the study of non-Hodgkin's lymphomas are treated separately.

At present, there are few agreed-upon standards for precision, accuracy, and interlaboratory comparability of leukemia analysis by FCM. Therefore, it is each laboratory's responsibility to establish instrument performance criteria and staining characteristics for its own specific reagents.

2 Introduction

This document represents the efforts of the CLSI Subcommittee on Flow Cytometry of Leukemic Cells to extend the guidelines for immunophenotyping by FCM to studies of leukemia and lymphoma. To this end, it builds upon the guidelines established in CLSI document H42—*Enumeration of Immunologically Defined Cell Populations by Flow Cytometry*, and several sections of this document are similar to those in H42. However, the subcommittee recognizes that the use of FCM to characterize cases of hematolymphoid neoplasia presents several specific problems that are not covered in H42. Issues specific to the study of hematolymphoid neoplasia that are covered in this document include:

- patient groups included;
- sample preparation techniques particular to neoplastic specimens;
- reagent panels employed;
- types of methodologic controls required and the necessary frequency of their use;
- rules and precautions followed in acquisition of data from neoplastic specimens;
- goals and methods of analysis unique to suspected hematolymphoid neoplasia samples, with emphasis on multiparameter analysis; and
- guidelines for interpretation and reporting of data.

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An overview of the differences between phenotyping neoplastic and non-neoplastic samples is provided in Section 4.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention (Garner JS, Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol*. 1996;17:53-80). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

4 Overview

In the previous edition, the original subcommittee outlined general principles that are useful for performing FCM on blood samples. Some of these principles are directly applicable to the study of neoplastic hematolymphoid samples. Because neoplastic cells differ in many ways from normal cells, and because the overall goals of phenotyping neoplastic hematolymphoid cells are different from those of lymphocyte subset enumeration, special procedures are necessary. This section outlines the most important differences; detailed information is then presented in appropriate sections to follow.

4.1 Goals

The principal goal of neoplastic hematolymphoid phenotyping is to determine the expression of lineage-differentiation and tumor-associated antigens on neoplastic hematolymphoid cells to aid in diagnosis and subclassification. This differs from that of blood lymphocyte phenotyping, whose aim is to enumerate antigenically defined lymphocyte subsets. Phenotypic characterization aids in the recognition and classification of the hematolymphoid malignancy. In some circumstances, the goal of FCM is to detect minimal involvement with hematolymphoid neoplasia for the purposes of staging or minimal residual disease (MRD) detection or disease prognosis. In these situations, sample preparation, data acquisition, gating strategies, and data interpretation are often different.

4.2 Quality Control Procedures

Instrument setup for phenotyping of hematolymphoid neoplasms is generally similar to that used for lymphocyte enumeration. However, in the case of hematolymphoid neoplasms, it is often extremely important to distinguish dim reactivity from either background autofluorescence or nonspecific staining. Section 11 addresses the importance of appropriate control reagents and also points out how to construct a panel of test reagents to include reagents that can also potentially serve as negative controls. Section 12 describes procedures for ensuring that the instrument is capable of resolving low-intensity fluorescence.

Several reagents used for phenotyping of hematolymphoid neoplasms may not react with normal blood lymphocytes, and special procedures for verifying their reactivity may have to be instituted.

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4.3 Sample Preparation

Although the methods initially developed for processing blood may be applicable to the study of bone marrow or other types of samples, special care is often necessary to ensure that the cell concentration is appropriate for the amounts of antibody being used and lysis methods do not impact on the viability of cells of interest. Fluids such as cerebrospinal fluid (CSF) and pleural effusions may be evaluated by FCM to detect involvement by leukemia or lymphoma. Lymph nodes or other tissues may be received and special procedures followed in order to produce a representative cell suspension.

4.4 Reagents

The reagent panels used for phenotyping of hematolymphoid neoplasms are more extensive than those used for enumerating lymphocyte subsets (see Section 10). Flow cytometric immunophenotyping of samples from patients with known or suspected hematolymphoid neoplasms requires detailed characterization of both neoplastic and normal cell populations, generally using antibodies directed against antigens expressed by those cells. Sufficiently detailed characterization requires sufficiently detailed antibody panels. Since appropriate antibody combinations and panels may vary according to various parameters, including patient age, disease prevalence in particular geographic areas, and specimen type, no specific panel or panels are described in this document, but several principles are stated. First, the best reagents for characterizing cases of acute and chronic leukemia are different. Second, many different types of multicolor reagent panels can be constructed. Laboratories are encouraged to design standard antibody combinations and panels that are appropriate to their own case mix, and become familiar with the patterns of reactivity seen with these reagents on either scatter vs. fluorescence or dual fluorescence plots.

4.5 Data Acquisition

Once stained with appropriate antibodies, samples suspected of containing hematolymphoid malignancies are introduced to the flow cytometer. Data about each cell's reactivity with each antibody, as well as its light scatter properties, are acquired and recorded in list mode for subsequent analysis and interpretation. Care should be taken to include all potentially neoplastic cell populations, as well as sufficient non-neoplastic cells to serve as internal controls whenever possible. Gated acquisition is therefore discouraged, unless the excluded events are known to be irrelevant. In some cases, neoplastic hematolymphoid cells may only represent a minor portion of the cells analyzed, and a large number of events must be acquired to gather sufficient data on the cells of interest. Due to technical advances, management of large data files is less cumbersome, and it is recommended that all signals be acquired. Because the data that are thus collected may be complex and contain several different populations, later setting of many different gates may be required when the data are analyzed. List mode is the default acquisition mode for all modern flow cytometers.

4.6 Data Analysis and Interpretation

4.6.1 Data Analysis

The goal of data analysis in hematolymphoid neoplasia is to determine which antibodies in a panel react with the malignant cells. To this end, descriptions of "percent reactivity" of several monoclonal antibodies are not informative, particularly in specimens that contain a mixture of malignant and non-malignant cells. Moreover, the conventional method of analyzing histograms—in which a cursor is placed at the boundary of a negative control and events in the test histogram to the right of this boundary are considered positive—is often not appropriate for analyzing distributions in hematolymphoid neoplasia because control histograms often show considerable overlap with test histograms containing low-intensity positives. Fluorescence intensity is an important variable.

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Data analysis in hematolymphoid neoplasia is different from that in enumeration of lymphocyte subpopulations. Comparison of histograms of single-parameter test data to controls may be appropriate for specimens in which the great majority of the cells are neoplastic. However, in almost all cases, substantially more information is gained when dual-parameter displays of data—either of correlated fluorescence or of scatter vs. fluorescence—are analyzed (see Section 13).

4.6.2 Data Interpretation

Once each of the antibodies in the panel is analyzed, and it is determined which ones react with the malignant cells in a specimen, some synthesis of the data should be done. When possible, a diagnosis should be given and simple description of results avoided. In some cases, the diagnosis and classification of hematolymphoid neoplasia requires morphologic assessment and/or molecular or cytogenetic demonstration of specific genetic abnormalities, in addition to flow cytometric immunophenotyping. Obviously, close communication between the FCM laboratory and the physician responsible for making the diagnosis is essential.

Results from FCM studies should be reported to give the composite immunophenotype and estimate of the number of the malignant cells in the sample. The interpretation should include the cell lineage of the tumor, its stage of maturation, and descriptions of unexpected results, such as aberrant antigen expression. Although some general rules can be laid out for deducing lineage based on patterns of antibody reactivity, it is not possible to provide a simple, unambiguous algorithm that can cover all possible outcomes.

5 Terminology

5.1 Definitions

3-parameter display – a graphic representation of data in which correlated values for three different parameters measured on the same cell are plotted.

allophycocyanin//APC – one of several phycobiliprotein-based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping; **NOTE:** APC has a molecular weight of roughly 80 000 daltons. Although optimally excited at 655 nm, there is sufficient excitation at 635 nm to produce a useable signal at 660 nm when excited with a helium-neon (HeNe) or diode laser.

analysis boundary – dividing line (cursor) placed on a histogram or dual-parameter display that discriminates events that are considered positive or reactive with a particular antibody from those that are negative or nonreactive; **NOTE:** An analysis boundary is most commonly set on a histogram obtained using a negative control antibody so that a fixed small proportion of events is considered positive. Traditional analysis boundaries are often inappropriate for analyzing neoplastic hematolymphoid samples.

analyte-specific reagents – antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended to use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens; **NOTE:** An analyte-specific reagent is the active ingredient of an in-house test.

autofluorescence – the intrinsic fluorescence of unstained cells, generally caused by pyrimidines and flavin nucleotides; **NOTE:** The level of autofluorescence is a function of the excitation wavelength and varies with the cell type analyzed and/or the state of cellular activation. Cultured cell lines, neutrophils, and macrophages usually demonstrate higher levels of autofluorescence with 488 nm excitation, and proportional lower autofluorescence with excitation at longer wavelengths. Autofluorescence can be decreased by specific sample preparation procedures (e.g., incubation with crystal violet).

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cluster of differentiation (CD) system – the identification of monoclonal antibodies with similar patterns of reactivity with human cells was the focus of numerous international workshops; **NOTE:** Each group of antibodies was assigned a CD number. Not all antibodies in a CD group react with identical portions (epitopes) of their target antigen. An antigen recognized by a given cluster of antibodies (e.g., CD4) is referred to as a "CD antigen" (e.g., CD4 antigen). An antibody that belongs to a given cluster is referred to as CD "x," with the manufacturer's nomenclature given in parentheses (e.g., CD4 [Leu3a]).

color compensation – a mathematical or electronic correction applied to flow cytometric data to account for the overlapping fluorescence emission from one fluorochrome in the wavelength region where the second is measured; **NOTE:** Compensation is achieved by subtracting a fraction of one measured fluorescence signal from each of the other fluorescent signals, the amount for subtraction determined from appropriate single-stained controls. The number of signals involved is equal to the number of fluorochromes (N) used and the number of compensation coefficients determined represented by an N x N dimensional matrix. The result is that each corrected signal reflects the emission of a single fluorochrome.

dual-parameter display (dot plot, contour plot, cytogram, two-parameter histogram) – a graphical representation of data in which correlated values for two different parameters measured on the same cell are plotted on an *x*,*y* grid.

dual-platform method – a method for the determination of absolute cell concentration using data derived both from flow cytometric measurements and a second instrument, generally a hematology analyzer; **NOTE:** The flow cytometer is used to obtain the fraction of the cellular subpopulation of interest present within a more abundant parent cell population, usually either total lymphocytes or total WBC. The absolute concentration of the parent population is provided by an independent assay of the sample on a second instrument. The product of these two measurements gives the absolute concentration of the population of interest. The disadvantage of the method is a compounding of the error inherent in each of the two measurements, and for this reason is judged inferior to single-platform methodologies.

epitope//antigenic determinant – that portion of an antigen against which the specific binding region of a monoclonal antibody reagent is directed; **NOTE:** Epitopes may be linear sequences of as few as six amino acids or conformationally determined sections of the antigen; each antigen typically contains multiple epitopes.

event – a single particle or cell identified by a flow cytometer.

external quality assessment – evaluation of the laboratory's performance on examination of samples of external origin for the purposes of determining adequacy of the laboratory's preexamination, examination, and postexamination activities (ISO/IEC Guide 43-1)[1]; **NOTE:** The main object is to establish between-laboratory and between-instrument comparability that is, if possible, in agreement with a reference standard (where one exists). External quality assessment schemes may be regional, national, or international. They may also be limited to the users of a particular instrument. It is sometimes also referred to as "proficiency testing," especially when the external agency is a regulatory agency.

fluorescein isothiocyanate//FITC – the most common fluorochrome for cell immunophenotyping; **NOTE:** Fluorescein conjugates absorb maximally at approximately 490 nm, close to the 488-nm emission of argon lasers, and emit maximally near 525 nm. Each conjugated fluorescein molecule adds a net negative charge to the antibody, and therefore may change its potential binding characteristics.

fluorescence intensity – a measurement of the amount of fluorochrome bound to a particle or cell; **NOTE:** Increasing intensity is reflected in a fluorescence signal appearing in a higher channel number. Under appropriate conditions, fluorescence intensity can be related to the number of binding sites a cell has for a particular fluorochrome-conjugated reagent.

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forward angle light scatter//low angle//FALS//FSC//FS – measurement of light at a low radial angle relative to the incident light source; **NOTE:** Measured values are a function of the cross-sectional area and refractive index of a cell or particle and the wavelength used for measurement. It is commonly used as an estimate of the relative size of a cell or particle.

gate – a set of parameters used to electronically select particular cells for evaluation; **NOTE:** Typically, a region of interest is defined based on one set of parameters (such as FSC vs. SSC), and other parameters (such as fluorescence) are evaluated only for cells within that defined region.

immunophenotyping – characterization of cells by the identification of cell surface or intracellular antigens, utilizing antibody reagents that recognize specific cell-associated molecules; **NOTE:** In this guideline, the cells that are immunophenotyped are lymphocytes and hematopoietic stem and progenitor cells.

internal control – cells of known phenotypic expression present in the sample analyzed; **NOTE:** Positive and/or negative expression and fluorescent intensity of such cells serve as process controls.

irreplaceable specimen – a specimen that is difficult to collect (invasive and/or surgically removed) and cannot be replaced by a repeat specimen, such as a lymph node or spleen; **NOTE:** Such specimens may be analyzed even when there is poor viability, since another identical diagnostic specimen cannot be obtained. This is in contrast to blood, which can be easily replaced with minimal impact on the patient's health. There are instances in which a blood sample may be considered irreplaceable, such as when recollection may delay diagnosis of an acute leukemia, or therapy may already have been initiated based on presumptive clinical diagnosis.

linear amplification – a linear amplifier (amp) produces a signal output proportional to the input signal amplitude; **NOTE:** For example, a linear amp could have output varying from 1 to 5 V as the input signal varies from 0.01 to 0.05 V.

list mode – a data storage format in which individual values of all parameters for each cell in a sample are retained, allowing maximum flexibility for subsequent analysis.

live gate – also called an acquisition gate or enrichment gating, this represents a set of parameters used to collect data on a population of cells for further analysis; **NOTE:** A live gate is typically set when the population of interest represents only a minor fraction of the total population, but when one or more parameters clearly distinguishes this population from the larger population of less important cells.

logarithmic amplification – a logarithmic amplifier (log amp) produces an output signal value proportional to the logarithm of the input signal value; **NOTE:** A four-decade log amp might, for example, have an output of 0 V for a 1-mV input; 2.5 V for a 10-mV input; 5 V for a 100-mV input; 7.5 V for a 1-V input; and 10 V for a 10-V input. Log amps are useful when analyzing samples containing cells whose measured parameters differ by orders of magnitude.

lysing agent – a reagent used to remove or lyse RBC from a sample in order to simplify the analysis of WBC; **NOTE:** Ammonium chloride in a bicarbonate buffer is the most commonly used lysing agent, although a variety of commercial preparations are also available.

peridinin chlorophyll protein//PerCP – a fluorochrome derived from the photosynthetic apparatus found in the dinoflagellate, *Glenodinium*; **NOTE:** PerCP is a protein complex with a molecular weight of approximately 35 kDa. PerCP has an excitation maximum around 490 nm, with an emission spectrum that peaks at 675 nm. Due to its photobleaching characteristics, PerCP conjugates are not recommended for use on stream-in-air flow cytometers and have been replaced in many applications by the tandem fluorochrome PerCP-Cy5.5 that is both brighter and has improved photostability.

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phycoerythrin//PE – one of several phycobiliprotein-based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping; **NOTE 1:** PE has a molecular weight of 240 000 kD. Although it absorbs light maximally at 545 nm, there is sufficient absorption at the 488-nm excitation by argon lasers to provide a usable signal in the red/orange (575 nm) emission range; **NOTE 2:** Denaturation of PE does not cause a spectral shift, but instead quenches the fluorescence. Aggregation of PE/immunoglobulin conjugates causes nonspecific binding.

primary antibody – an antibody that binds directly to the specific protein of interest.

procedure control – a specimen that is drawn, prepared, and stained with the same protocol used for patient specimens; **NOTE:** It is used (1) for instrument setup and monitoring for setting test-specific instrument settings and establishing color compensation settings; and (2) as a control specimen for sample and data analysis.

quality assurance – part of quality management focused on providing confidence that quality requirements will be fulfilled (ISO 9000)[2]; **NOTE:** The practice that encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. In the testing environment, this includes monitoring all the raw materials, supplies, instruments, procedures, sample collection/transport/storage/processing, recordkeeping, calibrating and maintenance of equipment, QC, proficiency testing, training of personnel, and all else involved in the production of the data reported.

quality control (QC) (internal) – the set of procedures undertaken in a laboratory for the continual assessment of work carried out within the laboratory and evaluation of the results of tests to decide whether the latter are reliable enough for release to the requesting clinician; **NOTE:** The procedures should include tests on control material and statistical analysis of patient data. The main object is to ensure day-to-day consistency of measurement or observation that is, if possible, in agreement with an agreed indicator of truth, such as control material with assigned values.

rare event analysis – analysis of cells present at low frequencies, such as stem cell enumeration or detection of minimal residual disease.

reference interval – the range of values obtained on specimens from normal controls assayed under the conditions used for patient specimens; **NOTE:** Assayed values that fall outside the reference interval are considered abnormal and may indicate a disease state. Relevant reference intervals are dependent on the assay methodology employed and on the composition of the patient population studied (e.g., with respect to age and sex).

resolution – the ability to discriminate between cells or particles that have different signal intensities; **NOTE:** The ability to resolve discrete populations is a function of biological factors (heterogeneity of signal within each population, difference in signal intensities between populations), as well as instrumental factors (sensitivity, measurement CV).

sample (patient) – one or more parts taken from a system, and intended to provide information on the system, often to serve as a basis for decision on the system or its production (ISO 15189)[3]; **NOTE 1:** For example, a volume of serum taken from a larger volume of serum (ISO 15189); **NOTE 2:** A sample is prepared from the patient specimen and used to obtain information by means of a specific laboratory test.

secondary antibody – an antibody that is labeled by fluorescent tag or biotin and binds to a primary antibody to allow its detection.

sensitivity – 1) in this document, the ability to distinguish signals of interest from background optical or electronic "noise" or biological signals, such as those due to autofluorescence or nonspecific reagent

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binding; **2)** the change in response of a measuring system or instrument divided by the corresponding change in the stimulus (modified from VIM93).[4]

side scatter (right angle [90° LS], wide-angle, side scatter [SSC], or orthogonal light scatter) – measurement of light at right angles to the incident light source; **NOTE:** This measurement is related to the internal and surface complexity of a cell or particle, such as cytoplasmic granularity, membrane irregularity, and/or nuclear shape.

single-parameter display//histogram – the frequency distribution of measured signal intensities (i.e., channel numbers) observed for cells within a population.

single-platform method – a method for the determination of absolute cell concentration with all data derived from flow cytometric measurements; **NOTE:** The determination of concentration requires an assessment of the volume of sample analyzed, and may be determined either directly by volumetric methods or indirectly by the addition of fluorescent beads at a known concentration. The latter method is more commonly used with the current generation of instrumentation. Single-platform methods are judged superior to dual-platform methods (see **dual-platform method** above).

specimen (patient) – the discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more quantities or characteristics, to determine the character of the whole; **NOTE:** The substance may still be referred to as a specimen if it was processed from the obtained specimen; thus, examples of specimens include whole blood and serum or plasma prepared from whole blood; saliva; CSF; feces; urine; fingernail clippings; hair clippings; tissue samples, even if embedded in a paraffin block; etc.

subclass control – an immunoglobulin of the same isotype (class or subclass) as the monoclonal antibody of interest, but without specificity for any known human antigens; **NOTE:** Most monoclonal antibodies used in FCM are either IgM or various subclasses of IgG (IgG1, IgG2a, IgG2b, or IgG3). This type of control is typically used to estimate Fc-mediated, nonspecific binding for antibody reagents of a given subclass.

tandem conjugate – a chemical complex of two fluorochromes arranged so that some of the energy emitted from the first is used to excite the second; **NOTE:** In practice, the most common tandem conjugates link phycoerythrin or other fluorochromes, which can be excited at 488 nm, to a fluorochrome that normally is excited at a longer wavelength than 488 nm, and that emits in the far red region. Examples of tandem conjugates are PE-Cy5, PE-Cy7, and APC-Cy7.

terminal deoxynucleotidyl transferase (TdT) – a nuclear enzyme found in immature cells, principally of the lymphoid lineage, but under some circumstances, in blast cells of myeloid lineage.

threshold – the level of signal above which a measured value is significantly different than background "noise"; **NOTE:** Threshold for instrument fluorescence sensitivity is the level of signal found for what is a nonfluorescent object. Threshold for determination of positive antibody staining is the level of signal found for cells not believed to react specifically with a given antibody reagent.

transport media – nutrient-rich media used to stabilize cells in a patient specimen for a sufficient period of time to allow transport to the laboratory and storage until analysis can be performed; **NOTE:** RPMI with fetal calf serum is an example.

viability dye – a dye that may serve to identify nonviable cells by virtue of its ability to penetrate cells with damaged membranes and subsequently bind to double-stranded nucleic acid; **NOTE:** Propidium iodide (PI) or 7-amino-actinomycin-D (7-AAD) are the most commonly used viability dyes. These viability dyes cannot be used as an indication of viability after fixation of cells by most methods because all cells become permeable and take up PI.

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whole blood lysis – a method of specimen preparation where antibody reagents are added to an aliquot of whole blood and, following incubation, a lysing agent added to remove RBC; **NOTE:** A subsequent wash of the sample may be used to reduce background fluorescence, but should not be performed if absolute counting beads are added. The primary advantage of the method is that it allows interaction of the antibody reagent with cells in a near native state and minimizes sample handling.

5.2 Acronyms and Abbreviations

ACD	acid citrate dextrose
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
amp	amplifier
BSC	biological safety cabinet
BSL-2	Biosafety Level 2
CSF	cerebrospinal fluid
CV	coefficient of variation
EDTA	ethylene-diaminetetraacetic acid
FCM	flow cytometry
FISH	fluorescent *in situ* hybridization
FSC	forward angle light scatter
IC	isotope control
kDa	kilodalton
LS	light scatter
mAb	monoclonal antibody
MCF	mean channel fluorescence
MRD	minimal residual disease
mV	millivolt
PE	phycoerythrin
PerCP	peridinyl chlorophyllin
PI	propidium iodide
QFCM	quantitative flow cytometry
RBC	red blood cells, erythrocytes
SSC	side scatter
TdT	terminal deoxynucleotidyl transferase
V	volt
WBC	white blood cells, leukocytes
WHO	World Health Organization

6 Safety

There are many biologic agents that may be present in blood and body fluids. Because any sample might contain an active agent of infection, which might result in morbidity or mortality if improperly handled, all specimens should be treated as though they are potentially infectious. “Standard precautions” should be observed.

6.1 Specimen Collection

All human body fluids and tissues should be treated as potentially infectious. Blood should be collected as outlined in the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*. Take care to prevent needlestick injuries. Needles should be discarded in a puncture-resistant container, and they should not be resheathed, purposefully bent, cut, or removed from the syringe.

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6.2 Safety Attire

Gloves and laboratory coats should be worn by all personnel who process specimens. If splashing or splattering of the specimen is anticipated, masks and protective eyewear should be worn to prevent exposure of eyes, nose, and mouth. Gloves should be changed and hands washed after completion of the work and before leaving the laboratory.

6.3 Biological Safety Cabinets

If possible, all work should be done in a biological safety cabinet (BSC, Class I or II). If this is not possible, at the very least all procedures with the potential for creating droplets or aerosols (e.g., vortexing, opening evacuated tubes) should be performed in a BSC (Class I or II).

6.4 Sample Containers

All specimens should be divided into aliquots and put into tubes that have caps, or microtiter plates that have covers (preferably sealed covers). The caps and covers should be in place except when adding to or removing from the containers.

6.5 Centrifugation

If specimens are centrifuged, care should be taken to avoid aerosol formation (i.e., in sealed vessels or inside safety carriers).

6.6 Pipetting

Pipetting by mouth is *not* allowed. One of the following techniques should be used: electronic pipettor, reverse pipetting, prewetting tip technique, and/or robotic pipetting system.

6.7 Sharp Devices

Use of "sharps" (e.g., needles, glass Pasteur pipettes, and glass containers) should be avoided as much as possible and disposed of properly.

6.8 Blood Spills

If a blood spill occurs, it should be decontaminated at once with an appropriate agent, such as 1:10 volume dilution of 0.71 mol/L sodium hypochlorite (i.e., undiluted household bleach), or appropriately diluted mycobactericidal disinfectants.

6.9 Waste Disposal and Specimen Inactivation

Collect used specimens and all disposable laboratory supplies, including needles and sharp elements, in puncture-resistant containers designed specifically for such materials. Dispose of by appropriate means (e.g., incineration or autoclaving), in conformity with local regulations.

Chemical techniques for specimen inactivation include mixing samples with a broad spectrum iodophor or bleach, retain for 24 hours, and dispose of properly. The bleach method cannot be used with ammonium chloride lyse solutions, as dangerous chlorine gas will be released.

For more information on the handling and disposal of medical waste, refer to the most current edition of CLSI/NCCLS document GP5—*Clinical Laboratory Waste Management*.

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6.10 Specimen Storage

It is preferable to analyze only samples in which infectious agents have been inactivated. Commercially available combined lysing and fixing solutions inactivate viral agents like HIV by three to five logs, while ammonium chloride had little effect.[5] For flow cytometric analysis, after the last centrifugation step in the labeling procedure, samples should be resuspended in a solution of buffered (pH 7.0 to 7.4) paraformaldehyde or formaldehyde (commonly 0.1 to 2.0%), and stored at 4 ^{o}C until analysis. The minimum storage time in paraformaldehyde/formaldehyde required to inactivate potentially infectious agents is not clearly established. Fixation is also used for stabilization of the sample so that it may be analyzed at a later date. Because specimen preparation and fixation techniques may vary depending on lysing agents and type/concentration of fixatives used, the minimum and maximum time the specimens should be stored in fixative varies. For validity of the analysis, the concentration of paraformaldehyde/formaldehyde and maximum storage time should be verified by the laboratory.

6.11 Unfixed Specimens

When unfixed specimens are run on the flow cytometer, the laboratory must be able to demonstrate that appropriate biosafety procedures are in place to minimize exposure to infectious material. Because aerosol generation in flow cytometers with a stream-in-air is a potential hazard, special attention must be exercised to operate the instrument in accordance with the manufacturer's biosafety recommendations.

6.12 Equipment Disinfection

The waste container on the flow cytometer should contain 1:10 of its volume of 0.71 mol/L sodium hypochlorite (undiluted household bleach).

Laboratory equipment should be properly disinfected with a solution known to inactivate infectious agents or a freshly made 10% solution of household bleach. It is particularly important that instruments be properly disinfected before repair or maintenance (see the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*).

7 Specimen Collection

7.1 Labeling of Specimen and Test Requisition

7.1.1 Requirements

All samples submitted for testing must be labeled immediately at the time of specimen collection with at least one unique patient identifier (in compliance with local regulations) and date and time of collection. When multiple specimens from the same patient are collected for analysis, the source of the specimens should be clearly indicated on the tube or vessel (i.e., blood and bone marrow from the same patient). In addition, a test requisition should accompany all specimens. This should include a unique patient identifier, presumptive diagnosis, age, sex, pertinent medication or recent treatment (including dates of chemotherapy or radiation), date and time of specimen collection, name of physician, and the source of the specimen (such as bone marrow aspirate or CSF). The test required should be on the specimen label or on the requisition accompanying the specimen. Information (such as WBC count, differential, morphological, cytochemical, molecular, and cytogenetic findings) should also be made available.

7.1.2 Specimen Age

Because specimen age is a critical variable in flow cytometric analysis, specimens must be labeled with the date and time of collection and processed as soon as possible. This is particularly important in cases of tumors with high proliferation rate (e.g., Burkitt lymphoma) or in patients where chemotherapy and/or

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radiation treatments were given. A 48-hour cutoff for specimen age is appropriate; however, **irreplaceable** specimens should not be rejected if they exceed the 48-hour specimen age requirements. Every attempt should be made to derive useful information from such samples. A statement regarding exceeded specimen age should be noted in the final report (see Section 14.5). Some antigens are more labile than others (e.g., CD 138 and CD 16) and may be preferentially affected with increasing specimen age. The potential false negativity of labile antigens should be noted when interpreting the antibody staining patterns in older specimens.

7.1.3 Biohazard Label

Specific labeling of specimens should be in accordance with national or regional safety standards. All specimens should be regarded as potentially infectious, and standard precautions must be followed.

7.2 Sample Collection Techniques

7.2.1 General

Fresh tissues and all body fluids (e.g., blood; bone marrow aspirates; and pleural, ascetic, or CSF) should be considered potentially infectious. Biosafety Level 3 (BSL-3) practices should be followed during the collection and handling of body fluids. Tissues and fluids cannot be fixed with formaldehyde or other fixatives, and should be transported to the laboratory as soon as possible. Every attempt should be made to provide the laboratory with fresh specimens for analysis. The choice of anticoagulant will differ depending on the sample (refer to Section 7.3 for anticoagulants of choice). Avoid extreme temperatures.

7.2.2 Venipuncture

The most current edition of CLSI/NCCLS document H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* should be followed for the collection of blood specimens.

7.2.3 Bone Marrow Aspirates and Cores

Specimens should be collected following standard medical practice for bone marrow aspiration. In the event of a "dry tap" bone marrow aspirate, a bone core specimen can be submitted for flow cytometric analysis. The bone core sample should be 2 to 3 cm in aggregate length and not fixed. It should be placed in a tissue culture media, such as RPMI in a sterile container.[6] Where multiple aspirations are performed (e.g., for morphology, FCM, cytogenetics), the secondary and tertiary aspirations frequently contain high proportions of blood. Samples should be prioritized accordingly. A comment on hemodilution should be included in the report.

7.2.4 Fresh Tissue Samples

Lymph node tissue, spleen, organ aspirates, and various other surgical and/or biopsy materials may be submitted for flow cytometric analysis. These samples are collected according to standard medical practice. Fresh (i.e., not fixed or chemically preserved) tissue samples should be placed in transport media (for external transport) or sterile saline-soaked gauze (for in-house transport) at the time they are taken, and kept moist throughout transport to the laboratory.

7.2.5 Other Body Fluids

Numerous additional sources of specimens are useful in the laboratory evaluation of the patient with neoplastic disease. Examples may include pleural fluid, ascites fluid, and CSF. These specimens should be collected according to standard medical practice. Once received in the laboratory, handle in accordance with standard precautions (see Section 3).

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7.3 Anticoagulants

The choice of anticoagulant depends upon specimen type, transportation, and storage requirements (see Section 8), and in some cases, method of sample preparation (see Section 9).

7.3.1 Blood Specimens

Ethylene-diaminetetraacetic acid (EDTA), acid citrate dextrose (ACD, solution A), or sodium heparin may be used. If a WBC count and differential are made from the same specimen used for FCM, EDTA is the anticoagulant of choice.

ACD samples are stable for 72 hours, sodium heparin samples are stable for 48 to 72 hours, and EDTA samples are stable for 12 to 48 hours. EDTA samples over 24 hours are suboptimal. Samples containing anticoagulants other than EDTA should be accompanied with an unstained blood film for morphological examination.

7.3.2 Bone Marrow Aspirates

Sodium heparin is the preferred anticoagulant for bone marrow samples. ACD anticoagulant is not recommended for bone marrow samples, as the ratio of sample to anticoagulant is not optimum, altering the pH and thereby reducing the viability of cells (see Section 9.4.1). Dipotassium EDTA can be used for the collection of bone marrow aspirates for flow cytometric evaluation; however, testing must be performed within 12 to 24 hours, and sodium heparin is required if the sample is split for cytogenetic testing.

7.3.3 Fresh Tissue Specimens

In general, no anticoagulant is used with tissue samples.

7.3.4 Other Body Fluids

Sodium heparin, EDTA, and ACD are appropriate anticoagulants when necessary, although samples preserved with ACD and held overnight may be vulnerable to low viability. If ACD is used, the appropriate ratio of fluid to anticoagulant should be used in order to not alter the pH, and therefore reduce viability. Anticoagulants are not appropriate for the collection of CSF. For additional information on collection of fluids, refer to the most current edition of CLSI document H56—*Body Fluid Analysis for Cellular Composition*.

8 Specimen Transport

8.1 Specimen Handling and Packaging

All specimens must be considered infectious and must be handled in such a way as to minimize risks for the laboratory worker and third-party carriers who transport specimens. Procedures for handling, packaging, labeling, and transporting specimens as defined by UN 602 must be followed.[7] Briefly, place specimen test tubes or vials (preferably unbreakable) in a leak-proof secondary container with sufficient absorbent material to absorb the entire contents of the vessel, if breakage should occur.

Carefully wrap or package glass slides to eliminate sharp edges and corners. The secondary container should have sufficient insulating capacity to maintain transportation temperatures at room temperature (18 to 22 ^{o}C). Insulated containers of one-inch thickness are usually suitable for this purpose.

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8.2 Specimen Integrity

Handling and transportation procedures must maintain the viability of the specimen. Specimen integrity is the composite product of anticoagulant, storage conditions (time and temperature), and sample preparation procedure. Specimens can be maintained at room temperature (18 to 22 °C) if they will be processed and fixed for flow cytometric analysis immediately after collection. Blood samples can be maintained at room temperature (18 to 22 °C) for up to 72 hours, samples are stable for 0 to 72 hours with sodium heparin and ACD anticoagulant, but sample quality may decrease after 24 hours with EDTA. Bone marrow aspirates (sodium heparin anticoagulant) may be maintained at room temperature for 24 hours. Fresh tissue samples should be placed in transport media or sterile saline-soaked gauze at the time they are taken, and kept moist throughout transport to the laboratory. If the specimen is held for any period of time, viability may decrease if tissue culture media are not used as transport media. However, autofluorescence may increase with the use of tissue culture media and consideration should be given to the impact of low viability vs. autofluorescence when choosing transport media. Cold (4 °C) storage may be useful for prolonged storage of some specimens, such as pleural fluids and cell suspensions prepared from tissues. Extreme temperatures must be avoided.

Many laboratories perform phenotyping of hematolymphoid neoplasms on transcontinentally shipped specimens. It is critical that the sample is shipped to the referral laboratory as soon as possible to minimize cell loss. It is not advisable to hold fresh tissue for flow cytometric analysis until after histologic sections are ready, as this delay could adversely affect the viability of the sample. Chemotherapy and/or radiation treatment may also drastically affect cell recovery. When specimen analysis is delayed by shipment to a remote laboratory, a freshly prepared and stained smear or tissue imprint and/or tissue section is useful for reference. An alternative is the addition of a stabilization fluid to the sample; this will allow specimen storage for up to ten days, so that retrospective analysis can be undertaken. Refer to Sections 7.1.2 and 7.3 for age of specimens and anticoagulants for analysis. Laboratories should demonstrate that, in their specific application, stored specimens give equivalent results to freshly processed ones.

9 Sample Preparation

The goal of sample preparation is to process a specimen from a patient with suspected hematolymphoid neoplasia into a representative sample suitable for introduction into the flow cytometer for sample analysis. All pertinent cellular and antigenic parameters for lymphoid, monocytic, and myeloid cells should be maintained. Irrelevant cells (e.g., RBC) that may slow or interfere with analysis may be removed. When neoplastic cells are present at low frequency, avoidance of their differential loss during cell preparation may be critical.

9.1 Visual Inspection

Observed abnormalities should be included in the report. Observable specimen problems are of two types:

- Those that indicate that the sample is altered or damaged: These call for immediate specimen rejection unless the specimen is irreplaceable.

- Those that suggest that specimen mishandling may have occurred: These call for further evaluation during sample preparation. Mishandling problems should be recorded because they may prove informative when preparing, analyzing, and interpreting data from the specimen.

9.1.1 Hemolysis

Hemolysis may indicate that the blood was exposed to conditions that can cause RBC lysis, which suggests that WBC may also be damaged and immunophenotypically altered. Interpretation of results

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should be approached with caution.

9.1.2 Clotted

Even a partial clot may cause selective loss or alteration of certain subpopulations. All clotted samples do not reflect what was present *in vivo*. Even if the laboratorian finds cells corresponding to one disease, there might be a second one undiagnosed. Clotted samples should be rejected unless irreplaceable, in which case every attempt should be made to obtain useful information. The clot can be removed and cells dispersed from the clot and filtered. A comment on the report should note that results may be affected.

9.1.3 Partial Draw and/or Low Volume Samples

For some anticoagulants (i.e., ACD), a partially filled tube may produce hypertonic conditions that are deleterious to cells. Depending on the magnitude of the underfill, the laboratorian may accept or reject the specimen. If the specimen is accepted, the laboratory should have previously demonstrated that the observed underfill does not affect final results, and the underfill should be recorded for consideration during preparation, analysis, and interpretation. Small sample volumes, such as those obtained from children's bone marrow, may be prevented from drying by for instance, immediate dilution in isotonic buffer once obtained.

9.1.4 Temperature Extremes

If the specimen tube was mailed to the laboratory, it may have been exposed to temperature extremes; however, examination upon receipt to determine if it is unusually warm or cold to the touch is not sufficient. For all deliveries, except those on the same day, it is recommended to include a minimum-maximum temperature sensor in the package to document whether or not extreme temperatures have been reached during shipment. Even if all other evaluation criteria are satisfied, this observation should be noted for further consideration during preparation, analysis, and interpretation.

9.1.5 Improper Specimen Labeling

All specimens should be labeled with a unique patient identifier, and date and time of specimen collection as specified in Section 7.1.1. If one is not present, then the laboratory should follow its established policy for dealing with unlabeled specimens and, if adequate identification cannot be obtained, the specimen should be rejected.

9.2 Selection of a Sample Preparation Procedure

In general, the more a cell suspension is manipulated or processed, the greater the opportunity for cell loss. Whole blood lysis method is the preferred approach. The advantage of an RBC lysis method is that it involves a minimum of manipulation. However, a lysis procedure assumes that all WBC subsets are equally tolerant to the lysis method used. This may not be the case with neoplastic hematolymphoid blood samples or marrow samples. It was shown that subsets of normal WBC are more susceptible to lysis than others, and that different lysis reagents have different effects. In some specimens, it is desirable to stain cytoplasmic antigens that require permeabilization of cells before staining.

9.2.1 Selection of Lysing Agent

There are several lysing reagents that are commercially available. These include water, Tris-buffered ammonium chloride, and hypotonic buffer. When using commercial reagents, always follow the manufacturer's recommended protocol, unless data are available that confirm that any modifications do not adversely affect results.

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9.2.2 Permeabilization Reagents

There are several permeabilization reagent kits available commercially. These should be used in accordance with the manufacturer's recommended protocol, unless data are available that confirm that any modifications to the protocol do not adversely affect the results. Choices of permeabilization reagents may be dependent upon the antigen being studied and the fluorochrome conjugated to the antibody.[8]

9.2.3 Lymph Node or Extranodal Tissue—Sample Preparation

This section will describe sample preparation of solid tissue samples and liquid samples, other than blood and bone marrow aspirates. These specimens include, but are not limited to the following:

- bone marrow core biopsies;
- lymphoid tissue biopsies;
- serous fluids;
- CSF;
- skin;
- mucosa; and
- fine-needle aspirates and core biopsies of tissue masses.

The goal of tissue transport, handling, and preparation of samples for flow cytometric (FCM) testing is to obtain a single cell suspension while maximizing the cell yield, maintaining the viability and integrity of the cells and preventing loss of abnormal cells of interest. Vigorous sample manipulation must be avoided to retain the cellular structure and antigenicity of the cells. The pH of reagents (transport media and buffers used in wash steps) must be strictly controlled. ***Rapid and gentle processing is imperative.***

Collection and transport of tissue samples can be a significant cause of potential problems with FCM analysis. Thorough communication with persons or facilities submitting samples regarding specimen requirements and acceptance guidelines is necessary. In addition, these samples require a more open-minded approach to rejection criteria. Due to the invasive nature of the procedures required for the collection of specimens for investigation of hematolymphoid malignancies (other than blood), recollection causes significant trauma to the patient. Therefore, *every attempt should be made to obtain useful information from samples submitted for analysis.*

A final word about the goals of quality sample handling is directed to documentation. Information about the sample source, collection date and time, and any pertinent, available clinical or diagnostic information should accompany the specimen. Documentation of receipt conditions and a trail of sample handling notes and signatures are valuable in the final steps of assessing the quality and usefulness of the FCM data, or in troubleshooting technique issues. Often knowledge of the sample source assists the laboratory in making decisions regarding steps involved in preparing the cells. Incomplete information regarding the sample, demographics (patient data), or the tests required may cause an unfavorable delay in appropriate testing.

9.2.4 Lymph Node or Extranodal Tissue Sample Submission

Strict guidelines about the required amount of sample do not exist because the yield of a sample depends on cellularity, cellular fragility, degree of necrosis, and ability to disassociate cells of interest (e.g., skin vs. lymph node biopsies) from tissue matrix. In addition, knowledge regarding the number of tests requested (and quantities required) for each sample may determine the size and/or quantity to submit.

Solid tissue samples should be kept moist in a sterile isotonic transport solution and rapidly processed to prevent dehydration, autolysis, and loss of proteins. For short-term storage (one hour or less), specimens may be maintained at room temperature. For in-house cases, this transport solution should be sterile

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saline, RPMI, or other isotonic buffers or nutrient media. For samples requiring overnight transit or holding until processing, solid tissue should be submersed in adequate transport media (RPMI supplemented with fetal calf serum and antibiotic) and maintained close to 4 to 16 °C (shipped with a cool pack, with specimen protected from direct contact with cool pack). Nonisotonic solutions or solutions containing fixatives are not acceptable.

When allocating portions of a single piece of solid tissue for different studies, the portion for FCM should be adjacent to the portion for histology, to ensure the abnormality is included and to prevent discordance with morphology due to sampling error. Fat and connective tissue should be minimized in the sample submitted for FCM. If a "dry-tap" or aspicular aspirate is experienced while collecting bone marrow, one or two 2-cm long core biopsies may be submitted and should be treated as a solid tissue. It is not recommended that testing for FCM be held until histology sections are available, as there can be a significant loss of viability necessary for adequate FCM resulting.

Fluid samples can be stored and transported as they are collected. If a large volume of fluid is collected (e.g., pericardial or pleural fluid), a representative sample can be used for FCM after the sample is well mixed. An anticoagulant, such as heparin, may be added to the fluids to prevent clotting, although this is not recommended for CSF.

The upper time limit for specimen storage, before testing, is highly dependent on the nature of the sample and the conditions during storage. Therefore, no strict guidelines exist. As a general rule, samples greater than 48 hours old should be carefully evaluated for sample characteristics that place the analysis in question.

9.2.5 Processing Lymph Node or Extranodal Tissue Samples

After initial sample verification (patient name, labeling, sample source, unusual conditions), a slide may be prepared for morphological review. The purpose of the initial slide is to ensure that cells of interest are present. Depending on the sample conditions, the following items may be used for morphology:

- touch imprint;
- cytocentrifuge slide (cytospin, especially useful for low-volume samples); and
- smear.

The next step is to prepare a single cell suspension for analysis on the flow cytometer. Distribution of the submitted sample and/or prepared cell suspension depends on the number and type of laboratory tests requested. If karyotyping is requested, a portion may be allocated for cytogenetics, under sterile conditions, before processing for FCM. If FISH and/or molecular testing are requested, the single cell suspension may be shared with the FCM testing. Always consider saving a minor portion for additional testing and verification.

Processing solid tissues for single cell suspensions can be performed in several ways. Each technique should have validations documenting that cells of interest are not lost, and that antigenic properties are not compromised. Mechanical disaggregation, which is generally preferred, is easy and rapid to perform and leaves the cells relatively unaltered. Disaggregation is achieved by use of commercial devices or with manual tools for slicing, mincing, and teasing apart the tissue (scalpel and forceps or syringe and needle). The manual cell suspension process should be performed over a wire mesh or if mechanical, followed by filtration through 50 μm of nylon mesh. A brief gentle vortex of the cell suspension can give better cellular yield. This is performed in an isotonic solution and the resulting cell suspension should be stored in this medium. It is reported that storage in cell culture media may increase autofluorescence; an isotonic sodium chloride solution or phosphate-buffered saline solution can be substituted.

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Enzymatic dissociation may be employed, especially in instances of tighter cell-cell adhesion, or when the cells of interest are difficult to release from adjoining tissue. This method usually requires some mechanical manipulation before incubation with a proteolytic enzyme (trypsin, pepsin). Enzymatic digestion can alter antigen expression and subsequent detection. DNase addition may help to reduce cell clumping in selected samples. Fine needle aspirate samples, particularly those from lymphoid tissues, usually do not require further disaggregation and can be directly stained after placing in an isotonic buffer/solution.

The primary goal when preparing cells for FCM is to ensure that the sample represents the tissue as close as possible to the native state. Minimizing sample treatment before staining greatly reduces the risk of losing cells of interest, and diminishes the possibility of increasing the number of nonviable cells. Single cell suspensions will continue to lose viability and *in vivo* structural characteristics over time. Therefore, *these samples must be processed gently and quickly* before staining and analysis.

Removal of RBC is considered a required, accepted manipulation before FCM testing. Morphological evaluation of the sample subjected to immunophenotypic staining can provide important quality assurance information. A cytocentrifuge slide of the cell suspension can verify that sample manipulation did not remove cells of interest or introduce any artifact material to the sample.

9.3 Adjustment of Cell Counts

Manufacturers' recommended amounts of antibody used generally assume normal ranges for the number of target cells stained. Normal WBC counts are between 0.2 to 2 x 10^6 nucleated cells per tube. Normal concentrations of antibody reagents may be insufficient to saturate all binding sites in densely cellular specimens, which may lead to false-negative results. Each laboratory must have a procedure to identify specimens with abnormal cell counts and correct for a target cell range of 0.2 to 2.0 x 10^6 total cells per tube, or according to the manufacturer's recommendations.

In cases of minimal residual disease detection, larger sample volumes may be required and antibody concentration should be adjusted to maintain appropriate antibody concentration.

9.4 Evaluation of Sample Viability

Each sample should be analyzed with close attention to the viability of the sample. Staining with monoclonal antibodies is ideal when the cell structure is maintained in its physiological state throughout testing. This is greatly altered by time from collection to analysis, pH, storage conditions, and sample handling. Because nonviable cells may nonspecifically bind to many antibodies and interfere with accurate immunophenotyping, it may be necessary to determine the viability of the sample submitted. This is particularly important in fresh tissue samples. Viability assessment must be performed on all tissue samples submitted for FCM, and if blood or bone marrow samples are received more than 48 hours after time of collection. Other situations that suggest the need for assessing viability are:

- when testing is delayed due to transport or other circumstances;
- when there is some doubt about how a specimen was handled;
- when the patient is undergoing chemotherapy or radiation therapy, rendering cells more fragile;
- in tissue samples; and
- when a highly proliferative neoplasm is suspected.

9.4.1 Methods of Viability Assessment

Viability assessment can be performed concomitantly with FCM evaluation of stained cells or by manual methods before staining. For "real-time" viability investigation, fluorescent dyes can be employed (propidium iodide, 7-amino-actinomycin D, ethidium monoazide) that bind to DNA. Ruptures in the cell

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membrane of nonviable cells will allow the uptake of the fluorescent dye. If this is done, it is essential to use unfixed specimens (see Section 6.11), since PI or 7-AAD may redistribute over all cells after fixation and storage. If alternative viability probes are used for postfixation viability discrimination, the laboratory must verify, using representative patient specimens, that such probes give results comparable to those obtained using a standard viability dye (i.e., PI, 7-AAD, trypan blue) in unfixed specimens. Whatever method is used, the laboratory is responsible for determining the concentration of viability dye to use to give optimal resolution between live and dead cells. If used in combination with monoclonal antibodies bound to other fluorescent compounds, nonviable cells can be removed from analysis with gating strategies, while evaluation of surface markers are made only on viable cells.

Trypan blue can be employed for a manual measurement of viability before staining. This requires manual cell counts on the hemocytometer. Neither procedure differentiates the nonviable cells as being solely the cells of interest or random cell loss.

9.4.2 Reporting of Samples With Low Viability

Samples should not be rejected solely due to low viability. When assessing viability, one should keep in mind that solid tissue samples often have lower viability than liquid samples. In addition, cell yield and viability are often lower in aggressive tumors (large cell process as opposed to small cell, indolent diseases), and large neoplastic cells are more fragile and susceptible to damage by sample treatment. There is no set rule that dictates a viability cutoff. A sample with low viability composed entirely of neoplastic cells can yield meaningful results. Guidelines are to reject samples with < 75% viability if it is not an irreplaceable sample (BM or tissue). If irreplaceable, and < 75% viable cells are present, issue a disclaimer statement about suboptimal viability but report any abnormal populations that are identified. If no abnormal populations are identified and viability is < 75%, consider rejection and requesting a new specimen. The rejection decision should be made by the interpreting pathologist with other clinical and technical features of the case taken into consideration. If reported, a notation in the final report should indicate suboptimal conditions possibly impacting the results. In samples with low numbers of viable cells, failure to identify a neoplastic process, even with nonviable cells gated out, must not be viewed as a negative result. Consideration and commenting on this fact should be made in the final analysis.

9.5 Sample Preparation for Immunoglobulin Staining

Anti-immunoglobulin antibodies are unique among reagents commonly used in clinical FCM in that they react with a substance that is present in high concentration in patient plasma and other body fluids. Accordingly, follow special procedures to ensure that the technique will detect immunoglobulin on the surface of cells. Moreover, many cell types will bind immunoglobulin nonspecifically via Fc receptors, and steps may be required to ensure that cell-associated immunoglobulin is intrinsic, and not extrinsic (or cytophilic).

9.5.1 Cytophilic Immunoglobulin

Adequate/effective washing with phosphate-buffered saline (PBS) before staining with anti-immunoglobulin reagents may be required to remove residual plasma or cytophilic immunoglobulin. Although washing removes these antibodies in most cases, a 30-minute to one hour incubation at 37 ^{o}C and washing again with buffered saline or tissue culture medium two more times, is more effective and may be required in some cases. This procedure may also remove other antigens, such as CD23.

The effect of cytophilic immunoglobulin can, in some circumstances, be mitigated by the appropriate construction of antibody panels. For example, B-cells generally have lower levels of Fc receptors than do monocytes, granulocytes, and some T-cells, so that combining anti-immunoglobulin antibodies with a pan-B antibody (called B-cell gating) can help ensure that immunoglobulin expression is only considered on the B-cell population and is strongly recommended. In this case, the non-B-cells will often

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demonstrate the presence of cytophilic Ig, though this will not interfere with analysis of the B-cells. In addition, for light chain analysis, simultaneous detection of kappa and lambda expression can also be useful because cytophilic Ig binding generally involves both kappa- and lambda-bearing immunoglobulin molecules, and such cells appear as doubly labeled (provided there is not a monoclonal immunoglobulin component in the serum). In contrast, B-cells express only one light chain and appear singly labeled.

9.6 Intracellular Sample Preparation

Detection of specific intracellular antigens is important in selected cases of leukemia and lymphoma. This is performed by first fixing the cells in order to maintain the structural integrity of the cells, and then permeabilizing the cells in order to allow the monoclonal antibody to enter the cell and attach to the respective antigen. If it is important to determine that the antigen is located exclusively intracellularly, special methods must be used. One option is to simultaneously stain for both surface marker and intracellular marker using separate aliquots. The surface marker aliquot must be negative and the intracellular antigen staining aliquot positive to prove exclusive intracellular location. In some cases with limited sample available, it is possible to stain for surface antigen before permeabilizing and stain for intracellular expression after permeabilization, using a different color than used to stain the surface. Again, surface staining should be negative for the intracellular antigens.

There may be a need to simultaneously stain surface markers and an intracellular marker (e.g., CD 33 and myeloperoxidase). In this situation, the surface markers are usually stained first followed by fixation, permeabilization, and then intracellular staining. With this method, it is critical that the surface marker fluorochrome is not affected by subsequent fixation and permeabilization. It is sometimes possible to stain for both surface and intracellular markers simultaneously after permeabilization. In this case, it is important that the surface epitope survive the fixation and permeabilization process. For intracellular staining, the fluorochrome must be small enough to enter the cell during the permeabilization step, although careful choice of permeabilization agent can usually allow staining with any fluorochrome. The washing step is particularly important in intracellular staining to remove fixative, residual permeabilization reagent, and nonbound fluorochrome-conjugated antibody. To avoid nonspecific results, antibody titration is extremely important when performing intracellular staining. The titration studies and validation of intracellular staining should be documented.

10 Immunofluorescence Staining of Surface and Intracellular Antigens in Neoplastic Hematolymphoid Specimens

10.1 Reagents

A fundamental principle of immunophenotypic analysis is that neoplastic hematolymphoid cells express surface membrane and intracellular antigens, which are related to normal cells of a particular lineage/maturational stage, and that can be detected with appropriate antibodies. Many different antibodies are used in the immunophenotypic analysis of neoplastic hematolymphoid cells. It is important to appreciate that most antigens are not absolutely specific for cells of a single lineage. Moreover, whereas normal hematolymphoid cells predictably express patterns of lineage- and differentiation-related antigens, neoplastic cells may exhibit unusual or aberrant patterns of antigen expression not seen in normal cells. In some instances, neoplastic cells may completely lack one or more antigens expected on normal cells of a particular lineage. In addition to such unusual or aberrant patterns of antigen expression, neoplastic cells may also exhibit anomalous antigen density, which is manifested as abnormal fluorescence intensity (either dim or bright), when compared with normal hematolymphoid cells. Although the immunophenotypic deviation of neoplastic hematolymphoid cells from normal cells may complicate diagnosis, such abnormal immunophenotypic properties enable the immunophenotypic detection of minimal residual disease (MRD) following therapy.

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As described above, complete immunophenotypic characterization of a neoplastic hematolymphoid cell population includes information about the presence, absence, and level (fluorescence intensity) of expression of a series of relevant antigens. Because of the inconsistent antigenic properties of neoplastic hematolymphoid cells, and the lack of absolute lineage specificity of most reagents, a panel of antibodies is required for immunophenotypic analysis. Such panels generally include both lineage-related antibodies, and reagents that are nonspecific as to lineage, but are nonetheless useful for immunophenotypic characterization. Although expert opinions differ with respect to the exact number of antibodies required to evaluate hematolymphoid neoplasms, it is generally accepted that limiting the number of antibodies in any given case may compromise diagnostic accuracy.[9] Furthermore, because antigen loss is not infrequent among hematolymphoid neoplasms, it is recommended to build a certain degree of redundancy into antibody panels. Finally, while there is some interlaboratory variation in preferred antibodies, in general, the most important reagents for the immunophenotypic analysis of acute leukemia are different from those that are most useful in cases of chronic lymphoid leukemia and lymphoma.

Ideally, the number of reagents should be sufficient to allow the recognition of the abnormal elements and all normal cells present in the sample. A reduced panel of antibodies may be used to confirm, classify, monitor, or stage a disease already diagnosed. Two approaches are usually applied to panel design: 1) use in a single stage an ample assortment of reagents that would allow a complete characterization of neoplastic and normal cells, regardless of any other consideration; and 2) apply a small number of antibodies for an initial limited analysis, followed by additional antibodies chosen on the basis of the initial results. This latter strategy is more economical but time-consuming, and requires decisions on antibody selection. In general, the larger the numbers of reagents, the higher the sensitivity and specificity of abnormal cell detection and characterization. A small number of antibodies may be applied using a "targeted" approach that is based on available clinical or morphological information. Although this approach may reduce cost, it can be risky in cases in which the supportive clinical information is incorrect or absent.

According to an international consensus,[9] a significantly large number of reagents are needed for a complete characterization of chronic lymphoproliferative disorders, tissue lymphomas, acute leukemias and other hematologic neoplasms. Participants recognized that at least 20 to 24 markers were required for complete characterization of acute leukemia. Similar numbers of markers were required for complete characterization of chronic lymphoproliferative disorders. In addition, new surface and intracellular markers have recently been proven to have diagnostic and prognostic utility.[10-14]

This already large assortment of reagents is likely to expand as new critical elements are recognized as playing relevant biological and clinical roles in hematologic and lymphoid neoplasia.

10.1.1 Cell Surface Reagents for Immunophenotyping Acute Leukemia

Cell surface antigen expression is generally used to assign a given case of acute leukemia into one of three broad general categories: B-lymphoid, T-lymphoid, or myeloid. Reagents may be divided into those that react primarily (though not exclusively) with these cell types. The following lists provide examples, but are not comprehensive, and the study of other antigens may be useful.

10.1.1.1 Examples of Cell Surface Reagents That React Primarily With B-Cells

- CD19 is expressed in nearly all cases of precursor B-cell acute lymphoblastic leukemia (ALL), although it is occasionally expressed in myeloid leukemia, as well.

- CD20 is less often expressed than CD19 on cells from precursor B-cell ALL; when present, it is typically of variable intensity.

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- CD22 is usually expressed on cells from precursor B-cell ALL, but its cell surface expression is often dim.

Surface immunoglobulin is generally absent in precursor B-cell ALL. However, surface immunoglobulin heavy chain without accompanying light chain was described in so-called "transitional precursor B-cell ALL," and in a small percentage of cases of otherwise-typical precursor B-cell ALL, both immunoglobulin heavy chain and monotypic light chain may be detected on the cell surface. Surface immunoglobulin heavy and monotypic light chain expression are characteristic of Burkitt lymphoma/leukemia and other more mature B-cell neoplasms.

10.1.1.2 Examples of Cell Surface Reagents That React Primarily With T-Cells

- CD2, the sheep erythrocyte (E) rosette receptor, is present in most cases of precursor T-cell ALL, but it is not specific, because it may also be expressed in different subpopulations of normal myeloid cells and in acute myeloid leukemia.

- CD3 and TCR are often absent from the surface membrane in cases of precursor T-cell ALL, but when present, they are highly specific for T lineage.

- CD5 is present in most cases of precursor T-cell ALL, but it is often dimly expressed.

- CD7 is the T-cell-associated marker that is most frequently expressed in precursor T-cell ALL, but it may also be expressed in acute myeloid leukemia.

Many cases of precursor T-cell ALL express the cortical thymic antigen, CD1a, and/or coexpress CD4 and CD8, indicating their relationship to immature (thymic) T-cells. Cases of precursor T-cell ALL with neither CD4 nor CD8, as well as cases with either CD4 or CD8, are also seen. Among these antigens, CD4 is probably the least specific, because it is found in many cases of myeloid leukemia.

10.1.1.3 Examples of Cell Surface Reagents That React Primarily With Myeloid Cells

- CD13 and/or CD33 are expressed in nearly all cases of acute myeloid leukemia. When these are expressed in the absence of the lymphoid-associated antigens noted above, this finding is strongly suggestive of myeloid leukemia. However, CD13 and CD33 expression are not specific for myeloid lineage, as these antigens are also seen in a significant minority of cases of precursor B- or T-cell ALL.

- CD117 is expressed in most cases of AML, and is relatively specific for myeloid lineage. However, occasional cases of $CD117^+$ precursor T-cell ALL, and rare cases of $CD117^+$ precursor B-cell ALL have been reported.

- Other myeloid antigens, such as CD11b, CD15, and CDw65, are generally expressed in myeloid leukemias that show morphologic evidence of differentiation.

- CD14, an antigen characteristic of normal monocytes, is frequently though not invariably expressed in myeloid leukemias that display monocytic differentiation. CD64 is brightly expressed by normal monocytes, and more dimly expressed by granulocytic cells. Bright expression of CD64 generally precedes that of CD14 in normal monopoiesis, and similarly, some cases of AML with monocytic maturation are positive for CD64 but not CD14.

- Glycophorin may be useful in the diagnosis of acute erythroid leukemias.

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- CD41 and CD61 react with platelet-associated glycoproteins, and may be useful to detect megakaryoblastic leukemias.

10.1.1.4 Examples of Miscellaneous Cell Surface Reagents

The common ALL antigen (CD10) is expressed in most cases of precursor B-cell ALL, and a significant number of cases of precursor T-cell ALL. This antigen may also occasionally be seen in AML.

While not lineage-specific, the progenitor cell antigen, CD34, is present in many cases of acute leukemia, and may help to distinguish abnormal from normal myeloid cells in heterogeneous populations.

CD58 is a nonlineage-related adhesion molecule that is expressed at predictable levels in normal B-cell precursors (hematogones), whose overexpression in many cases of precursor B-cell ALL makes it a useful reagent for MRD detection.

HLA-DR is expressed in virtually all cases of precursor B-cell ALL, and many cases of AML. This antigen is frequently absent from cases of T-ALL, and acute promyelocytic leukemia.

10.1.2 Reagents Used in Phenotyping Chronic Lymphoid Leukemia, Lymphoma, and Plasma Cell Dyscrasia

The task of phenotyping studies in mature/chronic leukemia and lymphoma is first to demonstrate the presence of a phenotypically abnormal/clonal population of B-, T-, and/or NK-cells, and second to classify it according to their maturation-associated features and aberrations. B-cell clonality can be determined based on restricted expression of immunoglobulin light chains; in a similar way, T-cell clonality can also currently be assessed based on the observation of a restricted usage of a T-cell receptor (TCR) TCRVα/TCRVβ or TCRVγ/TCRVδ family member. In contrast, phenotypic identification of clonality in NK-cell processes is not generally possible with current reagents. However, unusual patterns of antigen expression (such as strong positivity for CD5 on B-cells and CD56 on plasma cells, lack of CD3 and/or CD7 on T-cells,[15] and abnormally low CD56 expression on NK-cells[15]) may allow identification of abnormal cells. Once abnormal cells are identified, they may be further subclassified using additional B-, T-, and NK-cell reagents. The following lists provide examples, but are not comprehensive, and the study of other antigens may be useful.

10.1.2.1 Examples of Reagents That React Primarily With B-Cells

Because B-cell chronic leukemias, lymphomas, and monoclonal gammopathies are generally derived from mature B-cells or terminally differentiated plasma cells and are clonal proliferations, they express surface membrane and/or cytoplasmic immunoglobulin and show immunoglobulin light chain restriction. The intensity of expression of immunoglobulin and its localization (surface vs. cytoplasmic) may help distinguish among different types of B-cell tumors, at the same time, it may contribute to the identification of rare composite B-cell mature/chronic neoplasias with two different B-cell clones.[16]

Pan- or mature/chronic B markers, such as CD19, CD20, CD22, and CD37, are generally positive on mature/chronic B-cell leukemias and lymphomas, while rarely positive on T- and NK-cell tumors. One or more of the pan-B markers may be dim or negative on a mature/chronic B-cell leukemia or lymphoma, and panels should contain more than one pan-B marker.

CD10 is often expressed on B-cell lymphomas and leukemias derived from follicular center B-cells and Burkitt lymphoma, and it is characteristically absent from B-cell mature/chronic lymphocytic leukemia (CLL). Its association with overexpression or abnormally low/absent reactivity for bcl2 represents a characteristic feature of follicular lymphomas with (14; 18) and of Burkitt lymphoma, respectively.

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The distinction between mature/chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and mantle cell lymphoma (MCL) has prognostic significance, and has attracted the attention of numerous investigators. CLL/SLL is usually $CD23^+$, $CD5^+$ and exhibits dim surface immunoglobulin, CD20, CD22 and CD79b expression, with absent or dim FMC7. MCL is usually $CD23^-$/dim, $CD5^+$, bright CD20, and $FMC7^+$. CD103 and abnormally bright CD20 and CD22 are strongly associated with hairy cell leukemia. The clinical setting together with the forward light scatter (FSC) characteristics of these cells and the coexpression of CD25 and CD11c usually are sufficient to define this entity.

In B-cell mature/chronic lymphocytic leukemia, expression of some markers has prognostic relevance (e.g., reactivity for CD38 and/or ZAP-70 above certain thresholds [e.g., 20 to 30%]) in chronic lymphocytic leukemia associated with a poor outcome.

Positivity for CD138 and high intensity of expression of CD38 are the most characteristic markers of plasma cells. Clonal plasma cells from monoclonal gammopathy of undetermined significance and multiple myeloma frequently show aberrantly high expression of CD56, dim expression of CD45, and CD38 in the absence of expression of CD19, these representing the most useful markers for their characterization.[17] As the autofluorescence for plasma cells can be high, use of a negative internal control, such as CD3 staining of plasma cells, can be useful to determine negativity vs. positivity in malignant and normal plasma cells.

10.1.2.2 Examples of Reagents That React Primarily With T-Cells

Most mature/chronic T-cell leukemias and lymphomas express pan- or mature/chronic T markers, such as CD3, CD2, CD5, and CD7, in association with TCRα/β; in a small proportion of cases, neoplastic T-cells show TCRγ/δ in association with the other pan-T-cell markers, as usually found in the hepatosplenic T-cell lymphoma and some clonal expansion of large granular lymphocytes.

Because they are derived from mature/chronic T-cells, most mature/chronic T-leukemias and lymphomas express either CD4 or CD8, while in only a small proportion of cases do neoplastic T-cells either coexpress CD4 and CD8 or lack reactivity for both antigens. Expression of CD4 is associated with specific variants of T-cell lymphoma, such as mycosis fungoides/Sezary syndrome or angioimmunoblastic lymphadenopathy and adult T-cell leukemia/lymphoma associated with HTLV-I infection, while CD8 is expressed in other diseases, such as lymphoproliferative disorders of large granular lymphocytes.

CD5 is present not only on most T-cell leukemias, but is also expressed on a subset of B-cell leukemias and lymphomas, particularly B-cell CLL/SL.

T-cell proliferations can frequently be recognized as abnormal because of loss of a normal pan-T antigen; CD7 is the marker most commonly lost. In some cases, abnormal T-cell proliferations can be recognized by abnormal intensity of expression of a normal T-cell antigen, including CD2, CD3, CD4, and CD5, among others.

Although with the exception of CD5, aberrant expression of T-associated antigens is uncommon in mature/chronic B-cell leukemias and lymphomas, expression of CD2 and CD8 can occur in these malignancies.

10.1.2.3 Examples of Reagents That React Primarily With NK-cells

Natural-killer-associated markers, such as CD16, CD56, and CD57, are often expressed in large granular, lymphoproliferative disorders. As their normal mature/chronic counterpart, neoplastic NK-cells usually display expression of various receptors associated with NK-cell activity, as well as cytoplasmic expression of cytotoxic proteins, such as granzymes and perforin. However, since these markers may be

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also expressed by T-lymphocytes, in order to ensure the NK-cell origin of the expanded lymphocytes, these cells should lack expression of CD3 and TCR and their potential T-cell nature should be ruled out.

10.1.2.4 Examples of Predominantly Myeloid and Other Miscellaneous Reagents

- Certain myeloid-associated markers can react with subsets of lymphoid leukemias and lymphomas. For example, CD11c is often expressed in a subset of B-cell mature/chronic lymphocytic leukemia, and it is highly expressed in hairy cell leukemia and clonal cytotoxic T/NK large granular lymphocytosis.

- The stem cell-associated marker CD34 is essentially always absent from mature/chronic B, T, and NK-cell neoplasias.

- Activation markers, such as CD30 and (in the case of T-cells) HLA-DR, are often expressed in lymphomas derived from transformed lymphocytes. The exact significance of other activation-associated markers, such as CD25, that also identify a functionally different subset of regulatory $CD4^+/CD25^+$ T-lymphocytes, still remains to be established in the case of T-cell tumors.

10.1.3 Intracellular Reagents for Immunophenotyping Acute Leukemia

While most of the reagents commonly used for immunophenotypic analysis of acute leukemia recognize surface membrane antigens, the detection of certain cytoplasmic and nuclear antigens may be extremely helpful in acute leukemia. The following lists provide examples, but are not comprehensive, and the study of other antigens may be useful.

10.1.3.1 Examples of Intracellular Reagents

TdT is a nuclear antigen that is expressed in the great majority of cases of precursor B- and T-cell ALL, indicating their derivation from an immature precursor cell. It is also expressed in some cases of acute myeloid leukemia.

Cytoplasmic expression of myeloperoxidase is essentially limited to myeloid leukemia. Although myeloperoxidase is a specific marker of myeloid lineage, it is not sensitive, as it is absent in minimally differentiated AML, and may be undetectable or minimal in many cases of AML without maturation. In addition, myeloperoxidase is absent from myeloid leukemias, whose differentiation is neither neutrophilic nor monocytic (e.g., mast cell, dendritic cell lineages).

In precursor T-cell ALL (including those cases lacking surface CD3), cytoplasmic CD3 is almost always present, and its expression is probably the most sensitive and specific marker for T-cell derivation.

Similarly, in precursor B-cell ALL, cytoplasmic CD22 may be expressed, even in those cases with absent or dim surface CD22. In further distinction from surface CD22 expression (which may be seen in AML), cytoplasmic CD22 is specific for B-cell lineage.

Some investigators have suggested the use of cytoplasmic CD79a as a sensitive and specific marker of B-cell derivation. However, cytoplasmic CD79a expression has also been detected by FCM in some cases of precursor T-cell ALL. Moreover, detection of CD79a by immunohistochemistry was reported in AML. These findings call into question the specificity of cytoplasmic CD79a for B-cell lineage.

10.1.4 Assembly of Reagent Panels

As noted above, no specific panel can be recommended for leukemia phenotyping. Multicolor combinations of three or more fluorochrome-conjugated monoclonal antibodies are preferred. These

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combinations should be designed in a way that at least one marker (e.g., CD19 in B-cell tumors or CD45 in acute leukemias) is used in common in all combinations together with light scatter, for the reproducible identification of the cell populations. It is recommended that a laboratory choose a panel and gain experience with how normal and leukemic cells react when stained with that panel. Provided data are collected in list mode; there may be multiple possible ways of analyzing the same data (see Section 13).

10.2 Optimization of Staining Protocol

When commercially available reagents have a detailed protocol for their use, the protocol should be followed exactly. Any assay modification must have validation documentation to support that the change does not alter any of the predefined assay specifications. This is true for procedures for antibody staining, red cell lysis, and fixation.

In the event that a detailed protocol is not available, it is necessary for each laboratory to determine and document the optimum antibody titration and conditions of the staining procedure.

In red-cell-containing samples, erythrocyte lysis is the preferred method of RBC removal. Neoplastic cells have greater heterogeneity in density than normal cell populations, and density gradient purification may result in specific loss of abnormal cells.

The following variables have typically been examined by the manufacturers in arriving at their recommended procedure.

10.2.1 Temperature

Stain samples for immunophenotyping at room temperature (18 to 22 °C) in the dark.

10.2.2 Time of Incubation With Reagent

The time needed to reach saturation is affected by temperature, cell count, amount of the antigen under analysis in the sample, affinity of binding to antigen, and concentration of antibody. Typical staining times recommended by the manufacturer vary from 10 to 30 minutes. Where antibody panels are constructed from different manufacturers or have different incubation properties, the longest time period should be applied or laboratory data collected to ensure that optimum performance is achieved. Longer incubation does not necessarily equate with stronger signal. At room temperature, antigens such as CD3 and CD19 will begin to modulate in unfixed cells after ten minutes.

Protocols must be optimized and validated before patient testing. Once the optimal laboratory procedure is established, no deviations from the procedure should occur. If modifications are required, validation must be performed and documented before change.

10.2.3 Cell Count

The amount of antibody recommended for staining with most commercial reagents is sufficient to stain 100 µL of blood, assuming approximately normal WBC counts and that the antigen detected is only expressed on the WBC. Normal WBC counts in the sample should range from 0.2 to 2 X 10^6 nucleated cells (see Section 9.3 for adjustment of cell count).

10.2.4 Amount of Antigen in the Sample

When an antigen is present in the sample at very high amounts, either in a soluble form or on the surface of red cells, specific protocols may be required for its staining, even in the presence of adequate WBC or nucleated cell counts. Such protocols typically include red cell lysing and/or washing steps before

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staining. It is also possible that very high levels of cellular expression of an antigen can deplete the staining antibody and lead to a decreased fluorescence of the cells.

10.2.5 Reagent Concentration

Most commercial reagents have recommended amounts for staining a fixed volume of blood (or number of cells) in a fixed time. These recommended amounts are typically derived by determining the amount of reagent required to give the following:

- the maximum separation between the positive/negative populations; and/or
- the maximum fluorescence intensity of the positive population.

For each lot of a given reagent, the laboratory should verify the comparable positive/negative resolution and comparable positive-staining intensity with previous lots, using the laboratory's protocol for sample preparation. For such comparisons, staining of normal WBC and/or cell lines is recommended. Antibodies may be of sufficient concentration to provide adequate staining of cells when diluted past the manufacturer's recommended concentration, but this should be verified in titration experiments.[18]

For any deviation from the manufacturer's recommended staining conditions (e.g., time, volume, temperature, cell number, or sample type), the laboratory must determine the minimal amount (volume or weight) of antibody required to give optimal positive/negative resolution and optimal positive-staining intensity.

NOTE: Excess reagent may cause increased nonspecific staining of negatives, or it may cause quenching, resulting in decreased staining intensity and decreased resolution of positives and negatives.

11 Sample Quality Control

11.1 Verification of a Neoplastic Hematolymphoid Population

Bone marrow or blood samples submitted for immunophenotyping should, where possible, include information about the number and nature of neoplastic hematolymphoid cells present in the specimen. Morphologic examination of the cell population analyzed is useful to confirm that neoplastic hematolymphoid cells are present. In the case of lymph nodes, it may be desirable to review a cytocentrifuge preparation, to ensure the nature of the material present in the cell suspension correlates with that of the initial sample. In acute leukemias and most lymphomas, populations of neoplastic hematolymphoid cells are generally most easily resolved by correlation of light scatter properties and expression of certain antigens. Agreement between the flow cytometric phenotype of abnormal cells and the morphologic analysis is an important first step in assuring sample quality. In the chronic leukemias and indolent lymphomas, a novel population may not be resolved using light scatter characteristics, but morphologic confirmation is again important.

11.2 Negative Controls

Negative reagent controls are analyzed to establish the level of background fluorescence resulting from autofluorescence and nonspecific binding of primary and secondary antibodies. Panels of monoclonal antibodies composed of several immunoglobulin subclasses are typically used to characterize neoplastic hematolymphoid cells. Because the different subclasses show varying degrees of nonspecific binding, it is advisable to compare staining to that in negative controls for each subclass used, in order to estimate Fc-receptor-mediated nonspecific binding, even if steps were taken to block this (see Section 9.4). This can be accomplished by two approaches. When analyzing data, the negative cell populations present within the sample can serve as a negative reagent control. Alternatively, separate isotype negative controls can be used.

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Incubations with negative (isotype) control reagents can be set up in parallel to the panel of antibodies used in deciphering the neoplastic cells present. It is desirable that the negative controls run with the subclass be employed at the same immunoglobulin concentration as the test primary monoclonal antibodies. If cells are tested with direct antibody conjugates, it is desirable that the controls run with the subclass are of the same specific activity (fluorochrome to protein ratio) and conjugated to the same fluorochrome. The practice of pooling control reagents with different immunoglobulin subclasses is not recommended. In many instances, it is very difficult to find an appropriate negative control reagent, since different control reagents, even of the same subclass, may give variable staining intensities. This can be evaluated by staining a defined target cell with the subclass negative control and with monoclonal antibodies not reactive with that target.

In the second approach, negative staining with test antibodies of various subclasses in the panel is used to assess nonspecific binding. It is possible to coordinate panels that compare the binding of monoclonal antibodies of the same subclass that typically have mutually exclusive patterns of reactivity with subsets of hematopoietic cells (i.e., CD19 and CD33). In this way, test antibodies may also double as negative control reagents (e.g., staining with an antibody reactive with B-cells is a negative control in a T-cell process).

NOTE: It is emphasized that dim reactivity of test antibodies cannot be interpreted with certainty in the absence of appropriate negative controls.

Polyclonal antibodies are frequently used to detect the expression of cell-surface immunoglobulin molecules on neoplastic hematopoietic cells. Standardization of these reagents may pose difficulties because, unlike monoclonal antibodies, they are heterogeneous. It is necessary to analyze negative controls to determine the level of nonspecific binding of anti-immunoglobulin reagents. Manufacturers may prepare matched control reagents that have been optimally titered and absorbed. However, even when appropriate negative controls show no staining, a high level of false-positive staining with anti-immunoglobulin reagents may occur because these reagents may bind to cytophilic immunoglobulin bound to Fc receptors. Procedures for preparing the specimen to minimize this are described in Section 9.4.

11.3 Positive Controls

Positive controls are required to confirm that the methods used to prepare target cells, the immunologic reagents, and the staining procedures are performing optimally. Positive reagent controls and procedural or process controls perform this function.

11.3.1 Positive Reagent Control

The reactivity of immunologic reagents with defined target cells should be periodically characterized. Most of the monoclonal antibodies included in leukemia and lymphoma typing panels react with subpopulations of normal WBC. Reactivity of these immunologic reagents can be verified by analyzing blood WBC or other sources of positive targets, such as cryopreserved cells from lymphoid tissues. Reactivity of immunologic reagents can normally be verified during the course of sample analysis based on their reactivity with neoplastic hematolymphoid cells or residual normal cells in neoplastic samples.[19] The reactivity of immunologic reagents with control target cells of known reactivity should be verified within laboratory-defined intervals if a positive and negative target cell population is not encountered during this interval.

It may be necessary to identify an alternative source of positive target cells to test the antibodies. Such alternatives include the patient's normal bone marrow cells used as internal controls, appropriate human neoplastic hematolymphoid cells/cell lines, or commercially available preparations of preserved human WBC. If human neoplastic hematolymphoid cells are employed as target cells in positive reagent controls, they should be viably cryopreserved, maintained in liquid nitrogen, and properly thawed for each control

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analysis. The cell viability and recovery must be established and recorded each time the cells are processed, stained, and analyzed. Alternatively, human neoplastic hematolymphoid cell lines maintained in culture may constitute the panel of target cells of known reactivity. However, this is not practical for most laboratories.

Antibodies should also be analyzed to document their performance when reagent lots are changed. In this way, a new lot can be compared directly with the lot of reagent in ongoing use. Any difference in the fluorescence intensity of a known target population stained with a new lot of antibody should be investigated and recorded.

11.3.2 Procedural/Process Control

The most appropriate procedural/process control demonstrates that antibodies placed in the sample are capable of reacting under the test conditions. To this end, an antibody that has broad reactivity with hematopoietic target cells should be included in every typing panel. Examples of such monoclonal antibodies include those that recognize the leukocyte common antigen (CD45) and framework determinants of HLA class I antigens. Because the vast majority of samples contain T lymphocytes, it is possible to consider their reactivity with pan-T lymphocyte antibodies as evidence that the immunoassay is functioning. Virtually every sample submitted for flow analysis of leukemia/lymphoma contains normal hematopoietic cell populations. Antibody panels can be configured so that each tube provides known positive and negative controls. This approach has the disadvantage of not directly determining the ability of the target cells of interest to bind to a test antibody. This is a relevant concern in instances when hematopoietic malignancies are more fragile, and thus of lower viability, than normal lymphocytes.

Results in which the neoplastic hematolymphoid cells in a sample fail to react with any of the reagents in a test panel should be investigated and interpreted with caution.

Under some circumstances, a specimen from a normal donor that is drawn, prepared, and stained in parallel with the patient specimen may serve as a positive procedural control. It is not practical, or necessary, to analyze this type of procedural control sample daily or even weekly if the laboratory is active and if within-run positive and negative control results on a neoplastic hematolymphoid specimen demonstrate appropriate reactivity. However, for laboratories that do not process a high volume of neoplastic hematolymphoid specimens, such a sample should be prepared and analyzed when neoplastic samples are tested. An immunofluorescence-stained blood sample from a normal donor is recommended as the optimum material for determining instrument settings (see Appendix A). It is acceptable to use this same sample as the procedural control sample and to analyze the reactivity of monoclonal antibodies that identify the subpopulations of WBC that are represented.

12 Data Acquisition

A flow cytometer collects measurements of light scatter and fluorescence for each cell individually. *The primary goal of acquisition is to include all abnormal cells of interest in the data collected.* Following proper initial instrument optimization and rigorous daily QC (see Appendix A), certain parameters of instrument configuration for acquisition of data can be set to accomplish both general and specific needs. Immunophenotyping of hematopoietic malignancies represents a description of patterns of antigens expressed within or on the cells of interest, cytoplasmic and surface staining respectively. The role of FCM data acquisition is to provide data that allow interpretation of qualitative (positive and negative) and semiquantitative (intensity) analysis for these two types of stains. In addition, this discussion assumes a minimum of five-parameter analysis (three-color staining), or greater, since atypical cell populations are more accurately characterized by multicolor testing, which has become a standard of practice.

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12.1 Instrument Configuration

Acquisition of data from a sample involves a number of steps that ensure appropriate, high-quality data. The first steps for sample analysis involve proper configuration of the instrument. The variables in instrument configuration that can be user-defined include electronic settings, optical filters, signal amplification (linear vs. logarithmic), voltages, gain (range of signals measured), threshold settings (discriminates populations included in analysis), flow rate, compensation (for specific emission overlap), and the number of events collected. Protocols developed by each laboratory for specific procedures should address the instrument parameters that are user-defined.

Log amplification is generally recommended for fluorescence parameters in immunophenotyping heterogeneous populations, since it increases the range of detection. This is important for semiquantitative descriptions of "over- or under-expression" or "bright vs. dim." Light scatter parameters may be either linear or logarithmic.

Instrument threshold settings determine which events will have fluorescence and light scatter signals collected and retained. The threshold is usually set on FSC (size measurement) to exclude platelets, RBC, and debris, while not excluding the smallest WBC, lymphocytes, and nucleated RBC. Cytometers are capable of setting thresholds on one or more other parameters. Malignant cells may have a wide range of size and granularity. The initial data acquisition should be open, or all-inclusive, with subsequent analysis providing the ability to "gate" populations of interest for individual assessment of antigen expression. Certain protocols may set a "live-gate" on a parameter other than FSC. These strategies are generally used when enriching a specific population of interest (e.g., B-cells with abnormal antigen expression for clonality) or for rare event analysis.

For immunophenotyping, the high flow rate on most cytometers will produce a reasonable event rate (number of cells per second to pass the laser) of approximately 400 to 500 cells/second. The recommended minimum number of events is 10 000 to 50 000 events per collection tube. A higher number of events is usually required in lymphopenia or rare event analysis. When lower CVs are needed (e.g., for DNA content analysis) a slower flow rate may be necessary. If rare events must be analyzed, adjustment of the sample concentration can be done to increase the event rate without changing the flow rate. The maximum event rate that can be tolerated varies considerably among different cytometers, and should be carefully evaluated in each laboratory. High event rates can also lead to false double-positive events, due to coincidence of two cells in the analysis window of the cytometer; this potential should be considered when defining the maximum acceptable event rate.

When using electronic compensation, close attention to compensation settings is particularly important in the analysis of leukemia and lymphoma. Over-compensation can result in false-negative readings of antigen expression, and under-compensation can result in apparent (weak) expression of an antigen that is not actually expressed, both resulting in a potential for misdiagnosis. The number of fluorochrome conjugates used in each staining combination is generally three or greater. Overlapping of the emission signals of these various fluorochromes is dealt with by instrument or software compensation adjustments (see Appendix A). Tandem dyes can further complicate the compensation setting. Compensation is not independent of other instrument parameters. If any change is made to PMT voltage, gain, laser power, or optical filters, compensation settings must be verified and/or reestablished. Compensation should also be verified after preventive maintenance of an instrument.

Fixation of samples is recommended to minimize biohazard to the operator and to decontaminate instrument fluid waste collected. Additional decontaminating agents should be added to the waste containers (e.g., chlorine bleach). See Section 6.

A well-mixed sample is essential to achieve accurate representation of the cells present. This is done before instrument collection and often during data acquisition on some instruments.

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12.2 Enrichment for Cells of Interest

The general rule must be that exclusion of any significant subpopulation is safely done later during analysis, rather than during acquisition, because the latter would result in permanent loss of immunophenotypic information on the excluded cells. It is not possible to prescribe a uniform methodology to reliably exclude potentially contaminating cells and the cells of interest may be lost with acquisition gating. Counting errors for estimation of positive cells are theoretically proportional to the square root of the number of positive cells counted (i.e., if the population of interest comprised 5% of the total number of cells and 10 000 cells are evaluated, the expected count is 500 ± 22; if 1000 cells are evaluated, the expected count is 50 ± 7). In practice, at least 200 to 1000 cells of interest should be evaluated, but this ideal may not be achievable in patients with severely hypoplastic marrow. The ideal number of events to collect also depends on the relative purity of the sample for neoplastic hematolymphoid cells. In some cases, the population of interest may represent only a small subset of the total number of cells in the sample. For example, a small number of lymphoma cells may be present in a background of a large number of normal bone marrow cells. Under these circumstances, it may be appropriate to redefine acquisition gates for a particular stained tube to enhance the number of cells of interest. For example, when looking for B-cell lymphomas, samples stained with both a pan-B marker and kappa or lambda immunoglobulin light chain can be analyzed by setting a fluorescence gate and acquiring an enriched population of B-cells. If such a procedure is used, an initial acquisition should be performed as described in Appendix A and examined to identify alternate gating parameters. A second acquisition run using these new parameters can then be carried out to collect a data file enriched for the population of interest. An alternative and safer method is to acquire a sufficient number of total events to ensure acquisition of an adequate number of cells of interest.

12.3 Analysis of Negative Control Sample(s)

A decision to use negative controls should be determined for each protocol. Negative controls are most important when antigens are dimly expressed. Because all antibodies in a given combination are in different concentrations with different isotypes and F/P ratios, they may require their own isotype control. This is difficult in practice.

The US-Canadian Consensus Recommendations (1997) advocate the following statements regarding controls:

- *There is little or no need for a normal donor process control to be run with each batch of patient specimens analyzed.*

- *Specific isotype controls provide no useful additional information beyond unstained cells alone, or negative cells in selected antibody combinations.*

Specimens submitted for hematopoietic malignancy inherently contain a significant number of normal cells. Analysis of these normal cells permits an evaluation of the antibody-reagent performance. Panels, or combinations of antibodies used in evaluation, can be designed to provide both positive and negative controls by observing the normal cells in the sample. The negative staining of the cells of interest can be used to establish thresholds for determination of positive marker expression. Analysis of specific cell populations may require an unstained control for autofluorescence[17] (refer to Section 11 for sample QC).

12.4 Data Management

Data are collected in list-mode format. These data can be exported to external software for analysis, which is not restricted to software provided with the flow cytometer. The volume of these data will increase as instrument and software capabilities increase. A database must be designed to hold the data, allow retrieval of archived data (essential in establishing the presence of disease in subsequent samples),

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and should be available for the duration specified by accreditational and regulatory agencies. Knowledge of previous cases seen on a return patient must be a parameter of the database.

12.5 Consistency in Data

Intraspecimen data (i.e., those obtained on a single sample) using multiple flow cytometers should be consistent. FCM data, on a single patient sample, demonstrating fluctuation in light scatter properties can indicate sample preparation inconsistency, sample deterioration *in vitro,* or instrument fluctuation. Cell size and granularity should be constant in each tube prepared using identical sample preparation techniques (e.g., surface vs. cytoplasmic/surface staining). When multiple instruments are employed, instrument performance comparisons should be performed at least twice each year, but more often if indicated by data.

13 Data Analysis

13.1 Goals

The goals of data analysis in hematolymphoid neoplasms are as follows:

- Discriminate populations of abnormal, potentially neoplastic cells, from normal cells.
- Characterize the phenotype of the abnormal cells (size, antigenic profile).
- Enumerate discrete populations of abnormal and normal cells.

Unlike the case with analysis of lymphocyte subpopulations, many reagents, when applied to hematolymphoid neoplasms, give distributions in which low-intensity positives show considerable overlap with negatives. Analysis of such data is not straightforward. This problem is compounded further in specimens in which there are variable numbers of abnormal cells along with normal cells that may or may not label with the antibodies of interest. Ease (and to some extent accuracy) of analysis depends on several factors:

- the number of abnormal cells in the sample;
- the degree of heterogeneity of antigen expression in the abnormal cells;
- the intensity of expression of a given label, which in turn depends upon fluorochrome, instrument performance, and staining conditions (as well as the underlying pathobiology);
- the ability to separate abnormal cells from normal cells on the basis of LS characteristics and fluorescence; and
- the judicious design of reagent combinations, which, when analyzed using multiparameter analysis, can help distinguish normal and abnormal cells.

13.2 Identification and Analysis of Populations in Specimens

A critical step in the analysis is gating, which consists of selection of a discrete cell population of interest defined on the basis of light scatter and/or fluorescence characteristics. This should only be performed after analysis of all data.

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The recognition of abnormal cells in different tissues is based on the detailed knowledge of the light scatter and immunophenotypic characteristics (antibody binding patterns) of the normal cells in those sites. In each tissue or fluid, light signals from cells with similar phenotype create defined clusters in a pattern that is characteristic for that tissue or fluid and relatively constant. The presence of abnormal (e.g., neoplastic) cells results in changes in these patterns due to modifications in size, shape, or location of the normal cell clusters, or to the appearance of new clusters in areas of the graphs where normally few or no cells are present.

One or more of the following changes are usually detectable in hematolymphoid neoplasms:

- presence of cells with FSC and/or SSC different from that of normal cells;
- abnormal expansion of a single hematolymphoid cell subtype;
- expansion of cells with a rare phenotype;
- presence of cells with increased, decreased, or loss of expression of normal antigens;
- presence of cells with immature phenotypes in peripheral locations; and
- presence of cells with an immunophenotype not found in normal cells.

Initial analysis of data should be as inclusive as possible.

13.2.1 Examination of Light Scatter

Usually, the first correlated display examined is FSC vs. SSC. On this display, there may be only a single population of cells visible, or in some cases, more than one population may be identified. In some cases, there may be a major population of low SSC and an additional, well-resolved population with scatter properties of normal neutrophils. In the latter case, an analysis gate may be set on the non-neutrophil population. Any ambiguity in scatter pattern should always be resolved on the side of including questionable events in an analysis gate or analyzing using multiple gates. When multiple gates are set, they can be analyzed individually using the principles outlined below. Where possible, make separate descriptive statements about “large” vs. “small,” or “agranular” vs. “granular” populations. Intraspecimen variation of the position of populations defined by LS should be minimal for sample aliquots prepared in an identical manner and, if experienced, indicates a problem in either sample preparation or instrument function.

13.2.2 Examination of Scatter vs. Fluorescence

In cases in which there are mixtures of cell types present, correlated displays of either FSC or SSC vs. fluorescence may demonstrate distinct clusters of events that were not apparent from examination of scatter plots alone. Thus, populations may be gated according to size, expression of a particular antigen (e.g., CD20), or a combination thereof. Furthermore, gating may be multiple (e.g., cell size and two or more fluorescence parameters). Gating strategies are not universal and may vary depending on sample characteristics. However, such modes of analysis may be particularly helpful to distinguish between different subtypes of hematolymphoid cells. A few examples of markers that may help to distinguish different hematolymphoid cells are:

- CD45—shows gradient of expression from lymphocytes to erythroid cells (high level of expression in lymphocytes, absent from normal erythroid cells, and sometimes from blasts and plasma cells);
- CD3 and/or CD20—strongly expressed on mature T or B lymphocytes, respectively;

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- CD38 and/or CD138—strongly expressed on plasma cells;
- CD56—present on some large granular lymphocytes, and NK-cells;
- CD14—present on monocytes;
- CD15—strongly expressed on mature neutrophils and eosinophils; and
- CD71—strongly expressed on early erythroid cells.

It is important to note that these markers can also be expressed in a wide variety of abnormal cell populations, so it cannot be assumed that cells with the properties described above are normal in any particular sample. Nonetheless, because antigen density often differs between normal and abnormal cells, examination of FSC and/or SSC vs. fluorescence displays with markers such as these may help to distinguish normal and abnormal cells populations. Thus, for example, CD45 expression is usually of lower intensity on blasts in acute leukemia than it is on normal lymphocytes or monocytes, and is absent in nonhematolymphoid cells.

In mature lymphomas with a large cell component, forward light scatter gating may distinguish neoplastic cells from normal elements. Other strategies include gating on B-cells using a pan-B antibody, such as CD20, CD22, or CD19, in combination with other markers that help to characterize the neoplastic cells. B-cell gating in preparations containing a B-cell marker and antikappa and antilambda antibodies is extremely important in determining B-cell clonality. Similarly, a pan-T-cell antibody, such as CD3, may be used to characterize the phenotype of T-cell lymphoproliferative disorders. When compared to normal cells, these antigens may be over- or under-expressed in neoplastic cells, allowing for their selective analysis and detailed characterization, a strategy that is very useful in cases of partial or minimal neoplastic involvement.

Determination of fluorescence intensity for a given antibody is an important parameter for the interpretation of data and classification of many neoplasias. However, techniques for accurate measurements of fluorescence intensity have not yet been standardized for the clinical laboratory. For practical purposes, fluorescence intensities of neoplastic cells can be described in most instances as they relate to normal elements measured under the same conditions. This approach requires a clear appreciation of the range of fluorescence intensities of markers on different populations of normal cells for the particular reagents and instrument settings used.

Some of the practical considerations in gating strategies are demonstrated in Figures 1 through 6B.

13.3 Quantification of Antigen Expression

In addition to the presence of specific antigens on the neoplastic cells, the expression level or antigen density is also of diagnostic, occasionally prognostic, and potentially therapeutic importance. Accordingly, it is strongly recommended that the expression level of each antigen expressed be recorded during the data analysis. External standards, such as beads calibrated using MESF or ABC units, can be employed to accurately quantitate the level of antigen density on population of interest (refer to the most current edition of CLSI/NCCLS document I/LA24—*Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity*). Often, however, this is an unnecessary level of expense or complexity. Historic practices have included the use of semiquantitative estimates by the use of terms such as dim, moderate, and high or increased expression based upon the population position on a three- to four-log decade fluorescence scale. This subjective approach is affected by variables such as the fluorochrome attached to the reporting antibody reagent, gain settings to the fluorescence detectors, and the pH of the cell suspension solution. A preferred approach to antigen expression estimation is the comparison to known normal hematopoietic cell populations, which can be defined by either normal cell

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samples run at the same instrument setting or normal cells present in the patient sample in question. This approach works for most of the antigens used in practice, for example, normal T-cells can be used to define normal or moderate expression of CD3, CD4, CD8, etc.; normal B-cells can define CD19, CD20, and light chains; and neutrophils can be used to define CD10, CD16, CD13, and CD33. The advantage to this approach is that the quantification becomes meaningful to any laboratory, is independent of fluorochrome, environmental factors effecting fluorescence, and independent of instrument fluorescence detector setting.

13.4 Multiparametric Analysis

Due to software limitations, analysis is usually performed by examination of two variables at one time. In multiparametric analysis, the display analyzed is either a dot plot or contour map correlating the expression of two parameters.

After identification of the population of interest, analysis of the phenotypic characteristics is performed. For this purpose, sequential analysis of bivariate dot plots of all antibodies is frequently examined. Bivariate dot plots including LS and immunofluorescence staining are very useful in determining phenotypic characteristics of abnormal cells. In each of these bivariate dot plots, four areas are typically defined. These areas are as follows: events that bind neither antibody ("double negatives"), bind only one conjugated antibody, bind the other conjugated antibody, or bind both antibodies ("double positives"). Many software programs typically use rectangular quadrants based on setting perpendicular boundaries to define these areas. This may not always be suitable in many cases. Frequently, examination of such a two-color display reveals two or more distinct clusters of events, recognizable by the distribution of dots on a dot plot or by the contour levels on a contour map. In general, it is best to compare each recognizable positive staining cluster with clusters of negatively staining cells within the same test sample. When distributions containing low-intensity positives overlap with negative clusters, boundaries can at best, provide an estimate of percent-positive cells and may not be appropriate. A quantitative description of dim/heterogeneous positivity may be more accurate.

Some current commercial software products permit analysis of multiparameter data of mixed populations, using color to identify particular subpopulations. In these applications, the laboratorian can identify a discrete cluster of events on the one dual-parameter display in which they are most clearly separated from others, and then "see" where these events appear on other dual-parameter displays. The principal advantage of such methods is that it allows analysis of complex data without having to commit to a specific analysis gate. It also provides a powerful visual picture of the expression of antigens in combination.

Several examples of multiparametric analysis are illustrated in Figures 1 to 6B.

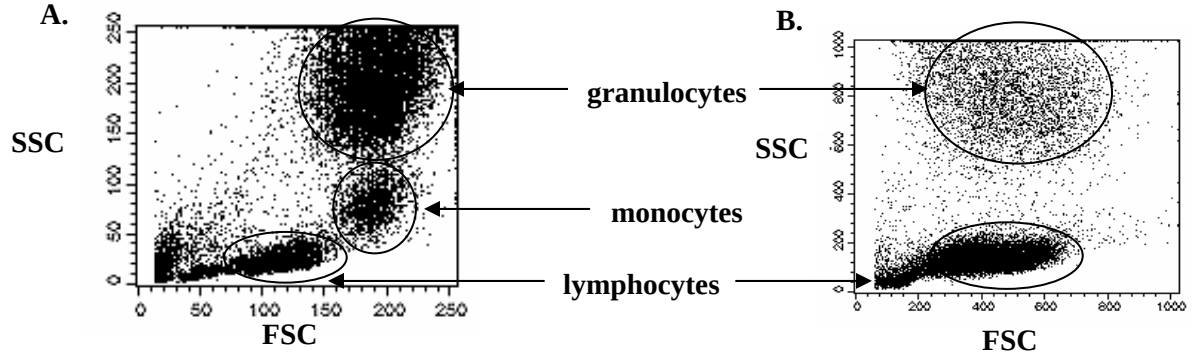

Figure 1. Linear Forward (FSC) vs. Linear Side Scatter (SSC) of Blood. (A) FSC vs. SSC in normal blood. Lymphoid, monocytic, and neutrophil populations are clearly demonstrated. (B) FSC vs. SSC in blood from a patient with hairy cell leukemia. There is a lymphocytosis, monocytes are absent, and neutrophils are decreased in number.

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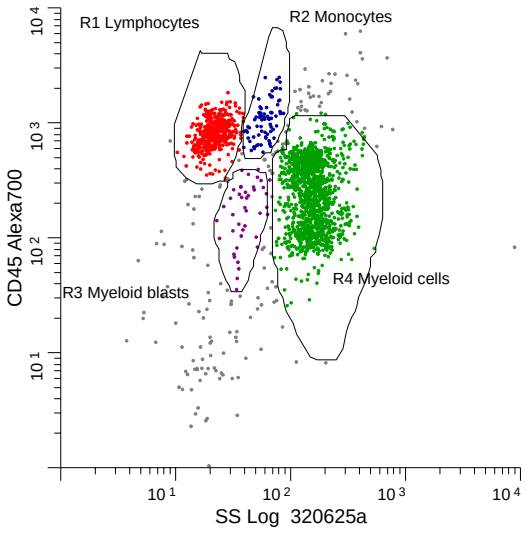

Figure 2A

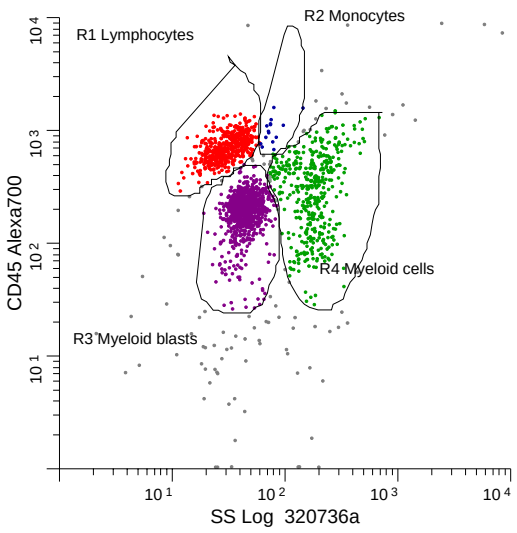

Figure 2B

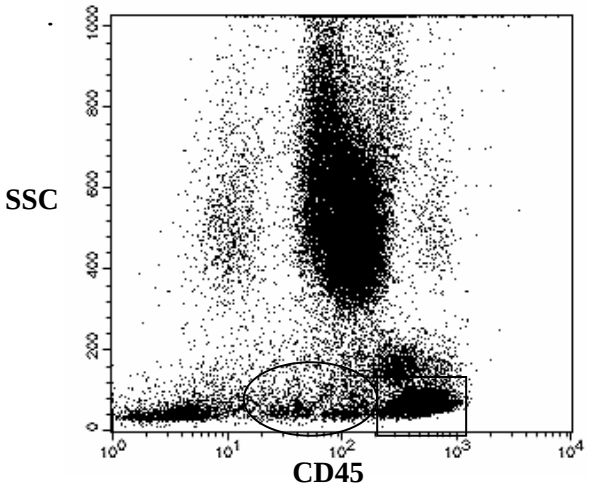

Figure 2C

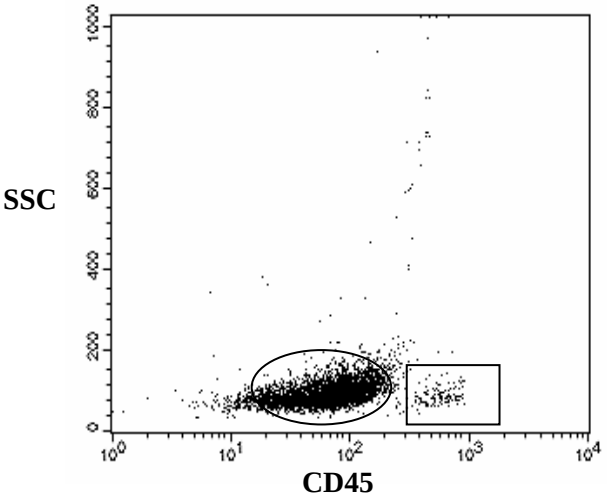

Figure 2D

Figures 2A to 2D. Analysis Based Upon CD45 and Side Scatter. (2A) Normal bone marrow—logarithmic right angle or side scatter (SS log) on x-axis and CD45 on y-axis. Note the separation of cell populations; R1 - mature lymphocytes, R2 - monocytes, R3 - myeloid blasts, R4 - maturing myeloid cells. (2B) Abnormal bone marrow sample with acute myelocytic leukemia—logarithmic right angle scatter (SS log) on x-axis and CD45 on y-axis. R1 - mature lymphocytes, R2 - monocytes, R3 - myeloid blasts, R4 - maturing myeloid cells. Note increased number of cells in myeloid blast region (R3). (2C) Normal bone marrow—CD45 on x-axis, linear right angle or side scatter (SSC) on y-axis. Mature lymphocytes in square, normal blasts in oval. (2D) Abnormal bone marrow—acute lymphoblastic leukemia. CD45 on x-axis, linear right angle or side scatter (SSC) on y-axis. Mature lymphocytes in square, lymphoid blasts in oval.

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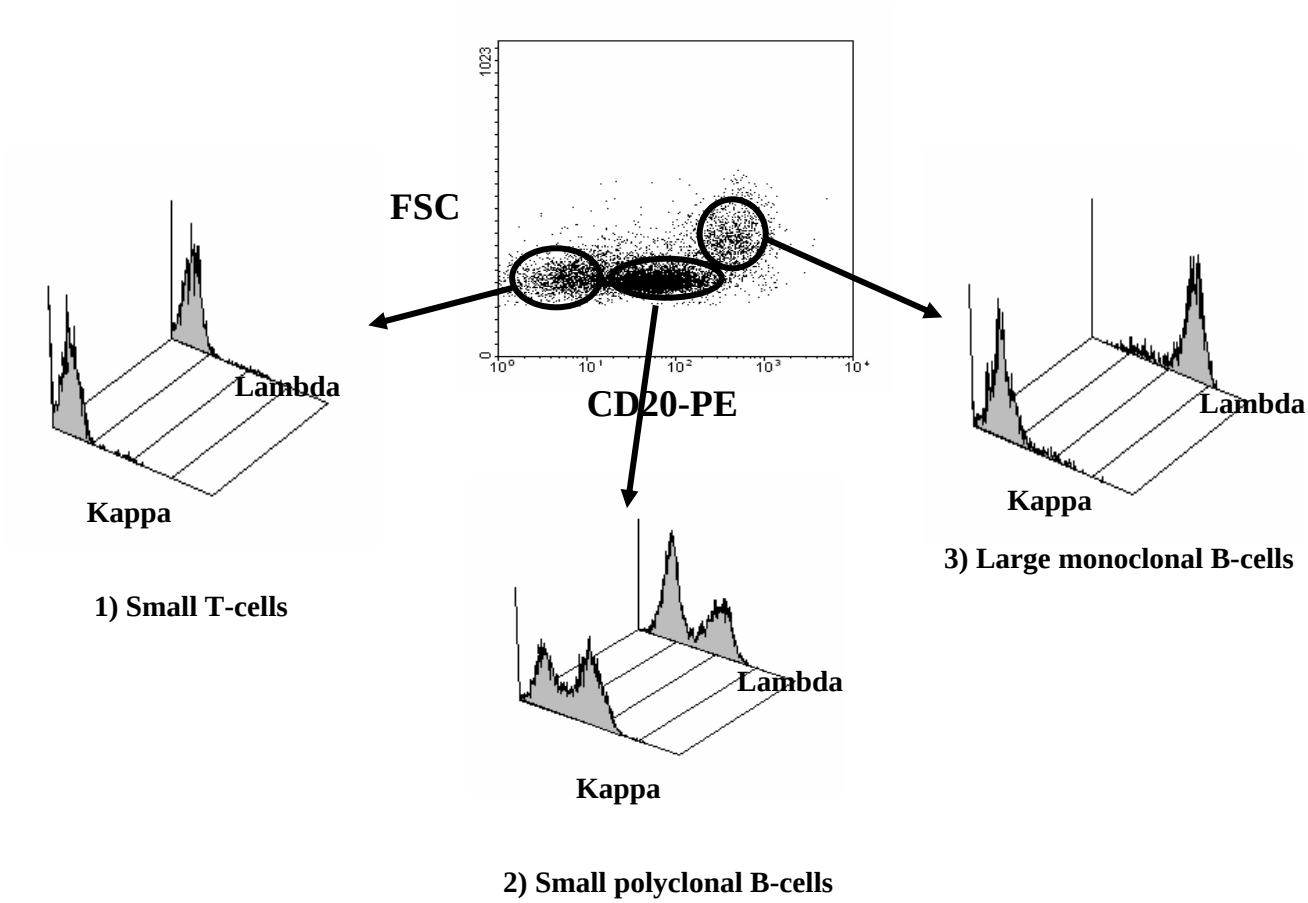

Figure 3. Focal Involvement of Lymph Node by Large B-cell Lymphoma. Cells from a lymph node were stained with a PE-conjugated anti-CD20 antibody and simultaneously with FITC-labeled antikappa in one tube and antilambda immunoglobulin antibodies in the other. The graph at the top center displays the analysis of cell size (forward scatter or FSC) and CD20 expression. There are three cell populations: 1) small (low FSC) $CD20^-$ T-cells that do not express kappa or lambda immunoglobulins; 2) small (low FSC) $CD20^+$ polyclonal B-lymphocytes with a mixture of kappa- and lambda-expressing cells; 3) larger and brighter $CD20^+$ monoclonal B-cells, which express lambda but not kappa immunoglobulins. The number of lymphoma cells in this sample is less than 10% of all cells.

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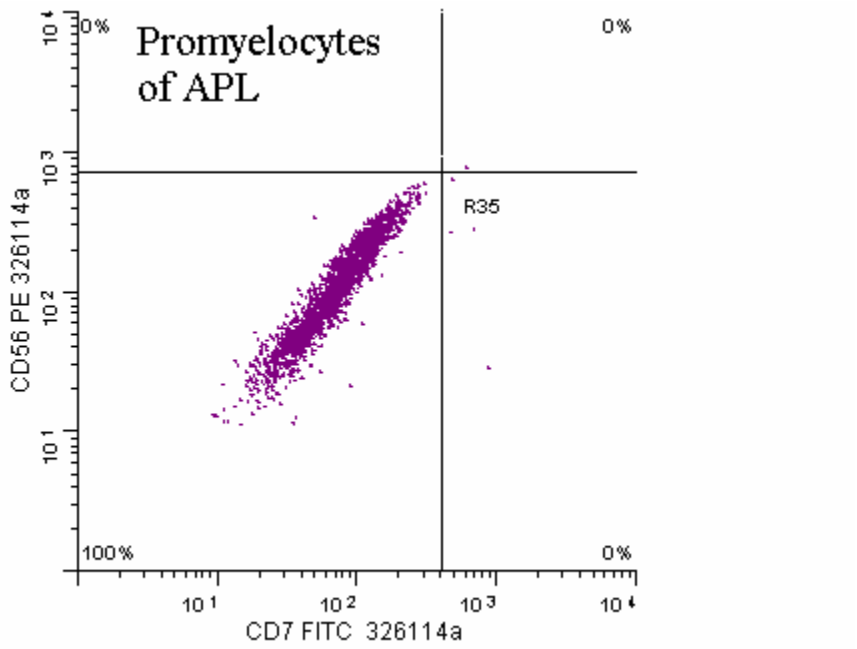

Figure 4. High Autofluorescence: Bone Marrow Involved With Acute Promyelocytic Leukemia. An analysis gate was drawn on the promyelocytes (based upon CD45 vs. side scatter) and only promyelocytes are shown. The promyelocytes have high autofluorescence. The quadrants are set to divide positive from negative. The promyelocytes are negative for both the CD7 and CD56 antigens. Note that rectangular cursor analysis is not appropriate to define antigen expression.

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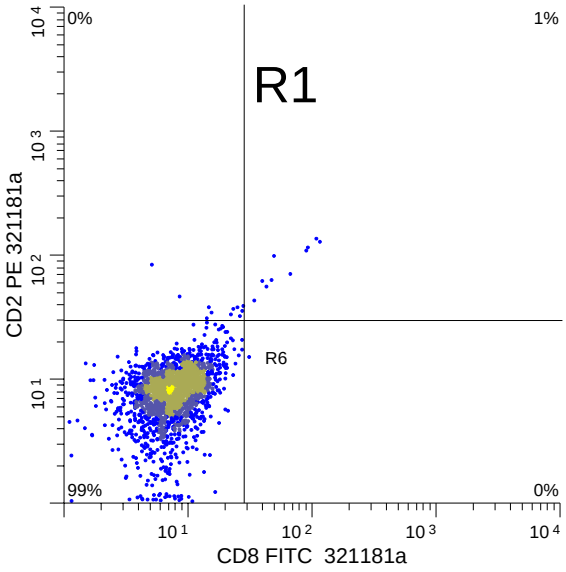

Figure 5A

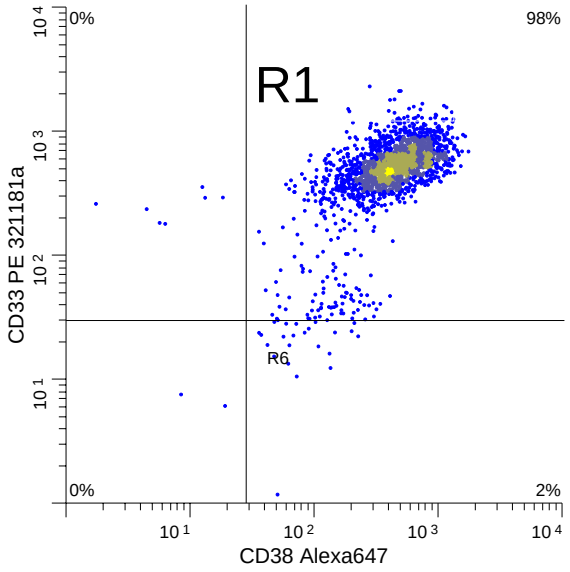

Figure 5B

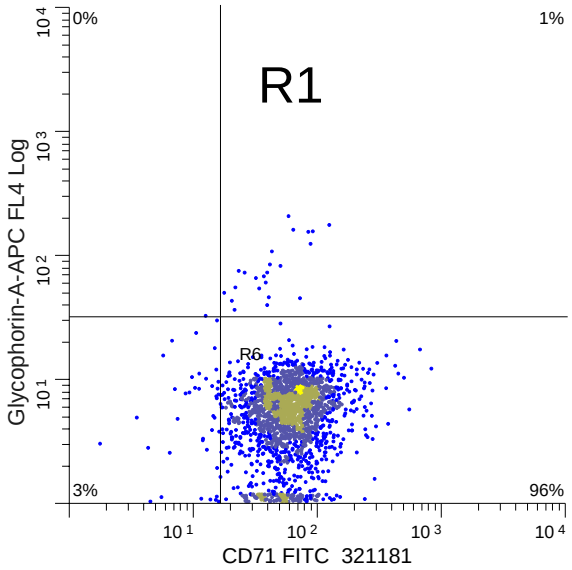

Figure 5C

Figures 5A to 5C. Myeloid Blast Population. A myeloid blast population is shown to demonstrate the following: (5A) a double-negative reaction for selected T-cell markers (CD2-PE on y-axis and CD8-FITC on x-axis); (5B) a double-positive reaction for CD33 and CD38 (CD33-PE on y-axis and CD38-Alexa 647 on x-axis); (5C) a positive reaction for CD71 and negativity for Glycophorin-A (CD71-FITC on x-axis and Glycophorin-A-APC on y-axis).

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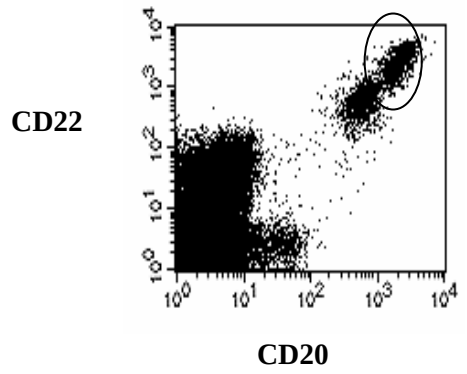

Figure 6A

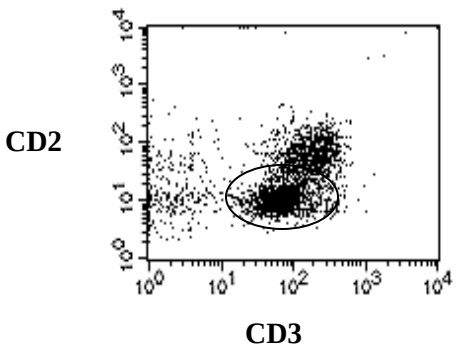

Figure 6B

Figures 6A and 6B. Abnormal Antigen Intensity in Hematolymphoid Neoplasia. (6A) Blood of patient with hairy cell leukemia. Hairy cells in oval have abnormally bright CD20 and CD22 compared to normal B-cells present. (6B) Fine needle aspirate of T-cell lymphoma. T-cell lymphoma cells in oval have abnormally dim CD3 and CD2 compared to normal T-cells present.

14 Data Reporting and Interpretation

14.1 Introduction

Because of the complex and varied nature of hematolymphoid neoplasms, interpretation and reporting of immunophenotypic data are frequently challenging, and no single algorithm accounts for all possible situations. The potential pitfalls are legion, and the impact of a misinterpretation on patient care may be significant. Therefore, it is strongly recommended that the individual responsible for data interpretation and reporting have adequate formal training and/or experience in the immunophenotypic evaluation of hematolymphoid neoplasms. Interpretation of clinical flow cytometric data derived from hematolymphoid cells requires an intimate knowledge of instrument function, reagents used, and sample handling and QC procedures at the laboratory performing the immunophenotypic analysis to avoid potential pitfalls that may lead to a misdiagnosis. In many (but not all) instances, the interpretation of immunophenotypic data is substantially augmented by integration with clinical and morphologic data; close communication between the individual responsible for interpretation and reporting of immunophenotypic data, and the person responsible for morphologic review (if they are not the same), and patient management may enhance the accuracy and utility of flow cytometric immunophenotyping. The practice of interpreting flow cytometric data by an individual without responsibility for the technical quality of the flow cytometric analysis itself should be discouraged. Interpreting and reporting data by individuals lacking both sufficient training and expertise in FCM and the ability to directly analyze list-mode files, is considered poor medical practice, and does not represent optimal patient care.

As clinical experience with immunophenotyping has increased, applications of the technique have expanded. In contemporary practice, bone marrow and/or blood samples from patients with anemia, leukopenia, thrombocytopenia, or leukocytosis are routinely submitted for immunophenotypic evaluation of a possible hematolymphoid neoplasm (e.g., acute leukemia, myelodysplastic syndrome, lymphoproliferative disorder, or plasma cell dyscrasia). Lymph node and extranodal tissue biopsies are commonly submitted in patients with lymphadenopathy or other tissue masses whose differential diagnosis includes malignant lymphoma. Body fluids (e.g., pleural, peritoneal, cerebrospinal) are also frequently submitted for the diagnosis or staging of patients with suspected lymphoproliferative disorders. In patients with an established diagnosis of a hematolymphoid neoplasm, follow-up specimens are evaluated to monitor disease progression and/or the impact of prior therapy. Finally, flow cytometric immunophenotyping in patients with a previously diagnosed hematolymphoid neoplasm in clinical

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remission may permit the detection of minimal residual disease (MRD). In many instances, the flow cytometric detection of MRD heralds subsequent clinical recurrence.

In recent years, much has been written about the interpretation of flow cytometric immunophenotyping data; several books are devoted largely to this topic. Thus, although an encyclopedic discussion of flow cytometric data interpretation and reporting is not feasible in a document of this size, some general guidelines are presented below.

14.2 Acute Leukemia

In current practice, the diagnosis and classification of acute leukemia require integration of clinical, morphologic, immunophenotypic, and genetic findings. Flow cytometric immunophenotyping serves several potentially useful functions in this process, including: facilitating precise enumeration of blasts in morphologically challenging cases (ensuring that the diagnostic threshold was exceeded); assigning lineage (i.e., B-lymphoid, T-lymphoid, or myeloid); providing information about the degree of differentiation; and in certain instances, anticipating underlying genetic abnormalities on the basis of the composite immunophenotype. Complete immunophenotypic characterization at the time of diagnosis also enables the identification of aberrant phenotypes, which may be exploited for MRD detection following therapy or evaluation of extent of disease at diagnosis.

Precise gating is a prerequisite for accurate immunophenotyping of a blast population in heterogeneous samples (i.e., those in which blasts do not comprise the overwhelming preponderance of cells). A precise gate ensures that the same cells are evaluated in different tubes, and that irrelevant cells with overlapping light scatter properties (e.g., erythroid precursors, lymphocytes, and monocytes) do not contaminate the blast gate, confounding interpretation. Although the immunophenotypic heterogeneity of acute leukemias necessitates modifying the location of the blast gate in multiparametric space in individual cases, a display of CD45 expression vs. SSC typically resolves blast populations well from other contaminating mononuclear cells. In designing antibody panels for the analysis of acute leukemias, therefore, it is useful to include CD45 in each tube.

14.2.1 Reporting of Immunophenotypic Data

The report should contain an estimate of the percentage of blasts in the specimen, and a description of the composite immunophenotype of the blast population (including antigen density as appropriate). For example:

“A population of blasts with dim CD45 expression and low SSC is identified, which comprises approximately 80% of cells in the specimen. The blasts are positive for HLA-DR, CD10 (bright), the progenitor cell antigen, CD34, and the B-lineage antigens, CD19, CD20 (dim, partial), and CD22 (dim). All myeloid and T-lineage antigens tested are negative.”

The alternate method of reporting positivity using percentages may be confusing; for example, in the following report:

HLA-DR 62%

It is not clear whether the HLA-DR value of 62% means that 62% of the blasts are HLA-DR$^+$, or that the sample contains only 62% blasts, all of which are HLA-DR$^+$. Therefore, when describing a hematolymphoid neoplasm, reporting of percentages of cells in the sample expressing individual antigens is not recommended.

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14.2.2 Guidelines for Interpretation of Immunophenotypic Data

14.2.2.1 Precursor B-cell Acute Lymphoblastic Leukemia

Virtually all cases of precursor B-cell ALL are positive for HLA-DR, with bright CD19 expression, and TdT is almost always positive. Surface CD22 is also commonly expressed, but may be dim. When present, expression of the more mature B-cell antigen, CD20, is often of variable intensity, and includes a subset of $CD20^-$ cells. CD45 fluorescence intensity is highly variable, and in a substantial minority of cases, CD45 may be negative or undetectable in some, or even all of the blasts.

CD10 is brightly expressed in most cases of precursor B-cell ALL. When CD10 is absent from a case of precursor B-cell ALL (particularly in an infant), a translocation involving chromosome 11q23 should be considered.

Most cases of precursor B-cell ALL are at least partially positive for CD34.

Myeloid antigen expression (most commonly CD13 and/or CD33) is detected in a significant proportion of cases of precursor B-cell ALL. In contrast with the bright expression of CD10 and CD19 seen in most cases of precursor B-cell ALL, aberrant myeloid antigen expression is commonly partial, and/or weak in these cases, and should not present a significant diagnostic challenge. Although myeloid antigen expression in precursor B-cell ALL may be associated with prognostic cytogenetic abnormalities (e.g., CD15 expression in infant ALL with 11q23 rearrangements), the weight of current evidence appears not to implicate myeloid antigen expression, per se, as an adverse prognostic factor.

When surface B-lymphoid and myeloid antigen coexpression renders lineage assignment uncertain, immunophenotypic analysis of intracellular antigens may permit resolution. Specifically, cytoplasmic CD22 is arguably the most sensitive and specific marker of B-lineage differentiation, while detection of myeloperoxidase (although uncommon in immunophenotypically ambiguous leukemias) would indicate myeloid lineage. Nonetheless, despite complete surface and intracellular immunophenotyping studies, lineage in a small minority of cases of acute leukemia will remain ambiguous (see below).

Surface immunoglobulin is generally absent from cases of precursor B-cell ALL. However, surface immunoglobulin heavy chain expression without accompanying light expression was described in so-called “transitional precursor B-cell ALL.” In addition, monoclonal surface immunoglobulin heavy and light chain expression (which is characteristically associated with a mature B-cell phenotype) has also been described in a small minority of cases of otherwise-typical precursor B-cell ALL. The leukemic form of Burkitt lymphoma (previously termed “L3” ALL in the FAB classification) also expresses monoclonal surface immunoglobulin heavy and light chains; however, this is a mature B-cell neoplasm with a characteristic morphology, which is negative for TdT and CD34, expresses bright, homogeneous CD20, and harbors translocations involving *MYC*.

14.2.2.2 Precursor T-cell Acute Lymphoblastic Leukemia

Surface CD7 is expressed in virtually all cases of precursor T-cell ALL; however, it is not a specific marker of T-lineage differentiation, and is not uncommonly seen in AML. CD2 and CD5 are at least partially positive in many cases of T-lineage ALL. Surface CD3 is less commonly expressed, but is comparatively specific.

Some cases of precursor T-cell ALL display a cortical thymic phenotype: CD1a+, with coexpression of CD4 and CD8; however, in other cases, CD4 and CD8 may be expressed individually, or may be absent.

CD10 and CD34 are more likely expressed in cases of precursor T-cell ALL with an immature (CD4-/CD8-) T-cell phenotype.

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CD45 expression in precursor T-cell ALL is generally brighter than that seen in precursor B-cell ALL, and in many cases, the CD45 fluorescence intensity of the blast population overlaps that of mature lymphocytes. This is important to consider when evaluating gating strategies in an individual case.

TdT is positive in most cases of precursor T-cell ALL; TdT^- cases are more likely to display a mature T-cell phenotype (e.g., $sCD3^+/CD4^+/CD8^-$).

HLA-DR is frequently absent, but weak and/or partial expression of HLA-DR is not uncommon.

As in precursor B-cell ALL, myeloid antigen expression (most commonly CD13 and/or CD33) may also be seen in precursor T-cell ALL.

Cytoplasmic CD3 expression is the single most sensitive and specific marker of T-lineage differentiation. This reagent is extremely useful in cases with surface expression of few T-lineage antigens, as well as those in which coexpression of surface T-lineage and myeloid antigens leaves uncertainty as to blast cell lineage (see below).

14.2.2.3 Acute Myeloid Leukemia

CD13 and CD33 are the antigens most commonly expressed in AML; when these are present in the absence of lymphoid-associated antigens, this finding is strongly suggestive of myeloid leukemia. CD117 is also frequently (although not exclusively) expressed in AML.

Other myeloid antigens are more commonly seen in cases of AML with morphologic evidence of granulocytic and/or monocytic maturation. Antigens associated with granulocytic maturation include CD11b, CD15, and CDw65, while bright CD11b, and/or CD14, and/or CD64 expression may be seen in association with monocytic differentiation. In this regard, it is important to note that the leukemic population in AML may include immunophenotypically distinct subsets. Thus, in acute myelomonocytic leukemia, a portion of the blasts may display a $CD11b(dim)^+/CD15(bright)^+/CD64(dim)^+$ immunophenotype suggestive of neutrophilic maturation, while the remainder expresses a composite immunophenotype indicative of monocytic differentiation: $CD11b(bright)^+/CD15(dim)^+/CD64(bright)^+$.

Acute erythroid leukemia includes erythroleukemia (erythroid/myeloid), and the less common pure erythroid leukemia. Erythroid precursors are characteristically $CD45^-$ or dim, positive for glycophorin, and express the transferrin receptor, CD71, brightly. However, the more immature erythroblasts in cases of pure erythroid leukemia may lack glycophorin. In this regard, it is worth noting that erythroid precursors, including erythroblasts, may be susceptible to lysis during sample preparation, and therefore may be underrepresented relative to myeloid cells.

Megakaryoblasts characteristic of acute megakaryoblastic leukemia may be recognized by the expression of platelet antigens, such as CD41 and CD61. A noteworthy pitfall in the immunophenotypic analysis of AML, though, is platelet adherence to myeloblasts, which may lead to an incorrect diagnosis of acute megakaryoblastic leukemia. Combining CD41 or CD61 with a more mature platelet antigen, CD42b, may facilitate distinction of megakaryoblasts (which are commonly, but not always $CD41^+/CD42b^-/CD61^+$) from mature platelets ($CD41^+/CD42b^+/CD61^+$) adherent to myeloblasts. A Wright-Giemsa-stained cytospin smear may also be useful in this situation.

HLA-DR is expressed in a majority of cases of AML. In those cases with absent or dim HLA-DR, though, it is important to consider acute promyelocytic leukemia associated with the t(15;17). Such cases are also often negative for CD11b, with heterogeneous CD13 expression, absent or dim CD15, bright, homogeneous CD33 positivity, and absent or partial CD34.[20]

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CD34 is positive in most cases of AML. Cases of AML with $CD34^-$ blasts (other than acute promyelocytic leukemia) are more likely to show some evidence of monocytic, erythroid, or megakaryocytic differentiation.

Expression of antigens typically associated with B- or T-lineage differentiation (including TdT) is not uncommon in AML. Indeed, in some instances, lymphoid antigen expression is associated with recurrent karyotypic abnormalities in AML [e.g., CD19 in t(8;21) AML, and CD2 in AML with t(15;17) or inv(16)]. However, in a small but significant minority of acute leukemias, the coexpression of lymphoid and myeloid antigens may preclude unequivocal lineage assignment (see below).

14.2.2.4 Acute Leukemias of Ambiguous Lineage

In a small minority (< 5%) of cases of acute leukemia, lineage remains uncertain despite thorough surface and intracellular immunophenotypic analysis. In the WHO classification, such leukemias have been designated "acute leukemias of ambiguous lineage," and are further divided into three subtypes:

- In *undifferentiated acute leukemia*, there is insufficient immunophenotypic evidence of differentiation to assign lineage. For instance, a leukemia that expresses HLA-DR, CD34, and CD7, but no other surface or intracellular antigens cannot simply be assigned to T-lineage, because of the lack of specificity of CD7.

- *Bilineal acute leukemia* refers to cases with two immunophenotypically distinct blast populations, each of which expresses a composite immunophenotype associated with a different lineage (e.g., both myeloblast and T-lymphoblast populations detected).

- In *biphenotypic acute leukemia*, there is a single blast population, which: (1) expresses markers associated with more than one lineage; and (2) cannot unequivocally be assigned to a single lineage on the basis of the composite immunophenotype. Strict adherence to the second requirement is essential in order not to misdiagnose otherwise uncomplicated cases of ALL with myeloid antigen expression, or AML with lymphoid antigen expression as biphenotypic acute leukemia. A scoring system based on the relative lineage specificities of several surface and intracellular antigens was proposed by the European Group for the Immunologic Classification of Leukemia (EGIL), and may provide some guidance in select cases.[21,22]

14.2.2.5 Minimal Residual Disease Analysis in Acute Leukemia

Response to therapy in patients with acute leukemia has traditionally been evaluated morphologically; patients with fewer than 5% blasts in the bone marrow are considered to have achieved "morphologic complete remission." However, small numbers of residual leukemic cells are nonetheless detectable using sensitive flow cytometric immunophenotyping or molecular methods in some patients who are in morphologic remission, and the detection of such minimal residual disease (MRD) following therapy may be an adverse prognostic factor in certain settings. Despite the prognostic significance of MRD detection in patients with acute leukemia, it should be recognized that at this time, treatments that impact the natural history of the disease have not yet been established. As noted above, neoplastic hematolymphoid cells commonly differ from their normal counterparts immunophenotypically. These immunophenotypic deviations include absence of antigens normally expressed, decreased, or increased antigen density relative to normal cells, and expression of antigens inappropriate for lineage or stage of maturation. Therefore, with appropriate combinations of markers, it is possible in multiparametric analysis to distinguish extremely small populations of leukemic blasts from normal marrow elements.

Several strategies for the immunophenotypic detection of MRD have been devised. Most commonly, cells are analyzed for antigenic combinations that are not detectable (or are detectable only at levels below the sensitivity of the assay) in normal bone marrow. For example, cells positive for both TdT and

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cytoplasmic CD3 (a composite phenotype seen in most cases of precursor T-cell ALL) are not detectable in normal marrow. Similarly, aberrant lymphoid antigen expression in AML, or myeloid antigen expression in ALL, serves to distinguish rare leukemic blasts from normal myeloid or lymphoid precursors, respectively. A prerequisite for this type of analysis is that the patient's leukemic blasts must express one or more abnormal antigenic combinations. In four-color analysis, abnormal antigenic combinations suitable for detection of MRD may be found in at least 90% of cases of ALL, and a smaller proportion of cases of AML.

An alternative approach to MRD detection is possible in precursor B-cell ALL. Normal B-cell precursors (hematogones) demonstrate a highly reproducible pattern of immunophenotypic maturation. If appropriate antibody combinations are used, greater than 90% of cases of precursor B-cell ALL will demonstrate significant deviation from the normal B-cell maturation pathway. In this type of analysis, differences in antigen density between leukemic cells and normal B-cell precursors are exploited. For instance, expression of CD10 and CD58 is often brighter than that of CD38 dimmer, in leukemic B-lineage lymphoblasts compared with normal B-cell precursors.

Each of these general strategies is capable of detecting one leukemic cell among 10 000 normal cells in ALL (0.01%) using four-color analysis; the sensitivity for MRD detection in AML may be somewhat lower. In principle, as the number of antigens evaluated simultaneously increases, the sensitivity of the assay should be enhanced. As a practical matter, though, the sensitivity of flow cytometric detection of MRD is limited by the number of total cells analyzed. As an example, in order to recognize a distinct population of 25 to 50 leukemic cells present at a frequency of 0.01%, a total of 250 000 to 500 000 cells must be acquired. Along these lines, in analyzing such rare events, it is essential to identify discrete clusters of cells in multiparametric space in order to distinguish MRD from other causes of unanticipated juxtaposition of fluorescent signals (e.g., nonspecific binding of labeled antibodies or fluorochromes).

An additional consideration in the immunophenotypic detection of MRD in acute leukemia is the stability of the abnormal composite immunophenotype over time. Immunophenotypic shifts following therapy or at relapse may be seen in ALL, and are quite frequent in AML. Such a change in immunophenotype may lead to loss of one or more abnormal antigen combinations, and yield a false-negative result. This possibility should be minimized, though not completely eliminated, through the use of multiple antibody combinations.

14.3 Mature Lymphoid Neoplasms

The various types of mature leukemia/lymphoma exhibit uniquely abnormal immunophenotypic characteristics ("fingerprints") that are helpful in their recognition. However, defining diagnostic criteria based on immunophenotyping for all possible lymphoid diseases is not within the scope of this document, and the reader is referred to publications specialized on this subject. Usually, interpretation of FCM results is integrated with morphologic and cytogenetic findings. However, flow cytometric immunophenotyping can help in resolving morphologic differential diagnoses and, for some diseases, it is possible to define criteria that can establish a diagnosis independent of microscopic findings. In general, the use of both techniques in combination greatly enhances diagnostic accuracy, and FCM laboratories not performing their own morphologic and cytogenetic examinations should make every effort to obtain this information from the same samples. It is also useful that the FCM, cytogenetic, and microscopic data be correlated with clinical and other pertinent laboratory findings, as well as other ancillary studies, as appropriate.

14.3.1 Mature B-cell Neoplasms

Mature B-cell neoplasms are clonal expansions of cells that, in the vast majority of cases, have productively rearranged the immunoglobulin genes. At this stage, B-lymphocytes produce only one of the two possible types of immunoglobulin light chain (kappa or lambda). In a B-cell lymphoma, neoplastic

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cells derive from a single B-cell and their light chain expression is restricted to either kappa or lambda. Occasionally, malignant B-cells express neither (crippled Ig gene). In contrast, in benign hyperplastic lymphoid tissue, the normal B-cells, having arisen from multiple ancestors, consist of a mixture of kappa and lambda bearing cells. Therefore, the presence of a B-cell population with a restricted expression of immunoglobulin light chain is, with few exceptions, consistent with a B-cell neoplasm. For this reason, immunoglobulin light chain expression, which is easily detectable by FCM, is one of the most important determinations in immunophenotyping. Although most B-cell neoplasms express surface light chains, plasma cell or early B-cell neoplasms, as well as some B-cell lymphomas, fail to show clear surface light chains. Additionally, cytoplasmic light chain can be used to define monoclonality, especially in plasma cell dyscrasias.

Having established that there is an abnormal proliferation of variably atypical lymphoid elements in blood, bone marrow, body fluids, lymph nodes, or other tissues, it is necessary to identify the lineage of such lymphocytes and to demonstrate they carry immunophenotypic abnormalities, which are characteristic of neoplastic as opposed to reactive and normal states. The neoplastic cells from both mature B-cell chronic leukemias/lymphomas and plasma cell dyscrasias frequently display aberrant phenotypes that allow the unequivocal distinction of neoplastic B-lymphocytes (e.g., overexpression of bcl2 on $CD10^+$ cells) and plasma cells (e.g., CD19 negative, CD56 highly positive, monoclonal intracellular light chain) from normal and reactive cells. One of the advantages of FCM is its ability to recognize monoclonal B-cells, even in the presence of polyclonal B-cells, debris, or among cells that have passively adsorbed immunoglobulins from the plasma *in vivo* (e.g., natural killer cells, activated T-cells, monocytes, or granulocytes), which may result in apparent surface light chain expression. Gates are set to isolate B-cells, and the light chain distributions of these cells are examined. Monoclonal B-cells may also display an abnormal size or aberrant antigen expression. By searching for these abnormal patterns, one can detect very small numbers of monoclonal B-cells, with sensitivity comparable or even higher than that of conventional molecular amplification of the Ig genes by PCR. It should be noted that neoplastic B-cells may express light chain epitopes not readily detected by all antibodies. Epitope differences may be important in light chain detection and more than one set of light chain reagents may be necessary for diagnostic accuracy.[23]

It is important to reemphasize that although immunophenotypic information is frequently useful in helping to subclassify chronic B-cell lymphoproliferative disorders and monoclonal gammopathies (e.g., coexpression of CD5 and CD23 in the context of dim surface immunoglobulin, CD22, CD20, and CD79b expression in chronic lymphocytic leukemia), caution should be exercised in making a diagnosis such as "typical chronic lymphocytic leukemia," or another clinical diagnosis in a report without coordinating the phenotypic information with other clinical and laboratory measurements. When correlation with clinical data and morphology is not possible, more ambiguous terms such as "monoclonal $CD5^+$ B-lymphoproliferative disorder phenotypically compatible with a typical chronic lymphocytic leukemia" may be more appropriate.

14.3.2 Mature T-Cell Neoplasms

The definition/classification of T-cell lymphoproliferative disorders based on morphology, immunophenotype, and molecular/genetic abnormality is generally less defined than mature B-cell neoplasms. Detection of mature T-cell neoplasia by FCM is more challenging than detection of mature B-cell neoplasia.

Mature/peripheral T-cell neoplasms are much less common than B-cell chronic lymphoproliferative disorders. Presence of neoplastic T-cells may be suspected because of an overwhelming T-cell predominance and/or the presence of mature-appearing T-cells displaying abnormal phenotypes. Phenotypic determination of T-cell clonality can currently be achieved through the demonstration of a restricted usage of a particular family member of the TCRVβ/TCRVα and TCRVγ/TCRVδ T-cell repertoire. The phenotypic diagnosis of clonality is better established by demonstrating expression of a

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single TCRVβ and/or TCRVα, or a TCRVγ and/or TCRVδ family member in phenotypically abnormal T-cells. The following abnormal phenotypic findings are suggestive of T-cell neoplasia:

- "anomalous" expression of T-cell-associated antigens (i.e., coexpression of CD4 and CD8); or
- deletion or abnormally low expression of one or more pan-T-cell antigens (i.e., CD3 dim, $CD5^-$, $CD7^-$).

Care must be exercised in interpreting minor populations of cells expressing these aberrant phenotypes because small numbers of activated/reactive cells displaying similar phenotypes can be seen in non-neoplastic conditions.

Cases that demonstrate an anomalous immunophenotype need confirmation as clonal through the phenotypic analysis of the TCRVβ/TCRVα and TCRVγ/TCRVδ repertoire or by other techniques. From these, Southern blot, or other DNA-based technologies such as PCR, is most useful to confirm the presence of a clonal process either on the whole sample or on purified T-cell populations.

Although immunophenotypic information can sometimes be useful in helping to subclassify chronic monoclonal T-cell disorders, when reporting, a conservative use of words is recommended. For example, a statement such as "atypical T-cell immunophenotype suggestive of Sezary syndrome" may be preferable to an outright diagnosis of "Sezary syndrome," when correlation with clinical data and morphology is not possible.

14.3.3 Mature NK-Cell Neoplasms

Among all the mature lymphoid malignancies, an NK-cell lymphoproliferative disorder is the most difficult entity to diagnose and to further characterize immunophenotypically. First, there is no significant difference between normal and abnormal NK-cells immunophenotypically, and for all practical purposes, there are no well-recognized subgroups of NK-cells, except the CD56 dim and CD56 strong positive subsets, to allow for determination of subgroup expansion as an indication of a possible neoplastic proliferation. Second, NK-cells do not express T-cell receptors and by definition, their TCR and immunoglobulin genes are in germline configuration. As such, flow cytometric analysis of TCR clonality cannot be used, and determination of clonal patterns of expression of NK-cell-associated receptors is still not proven as a reliable marker of clonality; in addition, molecular genetic techniques are of limited applicability. Because of these issues, the diagnosis of NK-cell neoplasms is best established by demonstrating an increased NK-cell number in a proper clinical setting. Additionally, identification of an expanded NK-cell population displaying abnormal phenotypes (i.e., $CD56^-$ NK-cells in adult blood) may be a helpful hint for identification of neoplastic proliferations of NK-cells. Identification of the NK-cell origin of a lymphoproliferative disorder should never rely on a single marker such as CD56, but on a full NK-associated phenotype (i.e., coexpression of granzymes, perforin, CD2, CD7, CD56, and CD16), after excluding a potential T-cell origin.

14.3.4 Minimal Disease Detection in Mature Lymphoid Neoplasms

Flow cytometric immunophenotypic analysis is an extremely sensitive method for detection of minimal disease in mature lymphomas and leukemias. Some low-grade lymphoproliferative processes, such as hairy cell leukemia or a lymphoproliferative disorder of granular lymphocytes, can present with cytopenias and increased infections, yet minimal blood involvement makes diagnosis very difficult unless flow cytometric immunophenotyping is used. Flow cytometric analysis is useful in staging of lymphoid neoplasia, where the demonstration of blood involvement may preclude the need for bone marrow biopsy. Detection of minimal residual disease (MRD) following therapy may provide useful prognostic information, and even herald relapse in patients without clinical or morphologic evidence of overt relapse. Nonetheless, as discussed above with respect to MRD detection in patients with acute

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leukemia, there are as yet no established treatments that impact the natural history of the disease in patients with mature lymphoid neoplasms.

Several strategies for the immunophenotypic analysis of minimal disease have been used, and differ in their efficacy based upon the disease being studied. Intensity of antigen expression, coexpression of antigens, and cell size can be used to target neoplastic B-cell populations for light chain analysis. Neoplastic B-cells frequently have abnormal levels of B-cell antigen expression, such as bright CD20 in hairy cell leukemia, bright CD10 in follicular lymphoma, and dim CD20 or CD22 in CLL. In addition, they may have a homogeneous size reflected by clustering in regions of small or large forward scatter (e.g., large cell lymphoma). By using FSC and SSC in conjunction with B-cell antigen expression, the neoplastic cells can be separated from the polyclonal B-cells based upon size and intensity of antigen expression. Malignant B-cells frequently express an abnormal pattern of antigens. For example, by targeting light chain expression in CD20 bright and CD11c bright B-cells, small populations of monoclonal hairy cell leukemia cells can be detected.[24-26]

Detection of minimal residual T-cell lymphoma is very challenging and usually the sensitivity is less than that observed in B-cell tumors. Because normal reactive T-cells are almost always present, analysis is complex and requires a multiparametric approach. Targeting a specific subpopulation (CD4 or CD8) in combination with antigen loss (e.g., $CD5^-$, $CD3^+$) and presence of atypical antigen (e.g., CD20 expression), abnormal antigen intensity (e.g., CD3 expression that is dimmer than normal) provides the greatest sensitivity. Confirmation of T-cell clonality by molecular methods can be useful in these cases.[27,28]

14.4 Myelodysplastic Syndrome (MDS)

Flow cytometric immunophenotypic analysis is useful in diagnosis and as a prognostic indicator in the myelodysplastic syndromes.[29-32]

14.4.1 Diagnostic Immunophenotypic Abnormalities in MDS

The myelodysplastic syndromes are a heterogeneous group of diseases characterized by dysplasia in one or more myeloid lineages. Although diagnostic criteria are established for MDS, a significant number of patients have blood and bone marrow findings that make diagnosis and classification difficult. Flow cytometric immunophenotypic analysis can help in diagnostic evaluation of such cases. There is no specific immunophenotype characteristic of MDS. Moreover, abnormalities seen in MDS may be seen in other disorders (such as PNH, megaloblastic anemia, and growth factor effect). Problems with sample integrity may artifactually produce such abnormalities. Multiple immunophenotypic abnormalities are common in MDS, and it is the pattern of multiple myeloid immunophenotypic abnormalities that allows one to distinguish MDS from other disease processes. The characteristic multiple immunophenotypic abnormalities can only be detected by incorporating a large number of antibodies in a multiparameter four-color-or-greater panel. The data can only be interpreted upon the background of an extensive knowledge of the normal patterns of antigen expression during myeloid differentiation. The abnormalities typically detected include antigenic asynchronous maturation, abnormal intensity of antigen expression and expression of abnormal low side scatter (due to hypogranular granulocytes), and antigens such as lymphoid lineage antigens in myeloid precursors.

14.4.1.1 Antigenic Asynchronous Maturation in MDS

Myeloid maturation is characterized immunophenotypically by the expression of a series of specific antigens at precise intensities in a tightly controlled sequence. Flow cytometric immunophenotypic analysis can detect MDS cells deviating from this rigid normal pathway.

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Examples of flow cytometric immunophenotypic antigenic asynchronous maturation patterns observed in MDS are as follows:[29-31,33,34]

- abnormal pattern expression CD16 vs. CD11b by maturing granulocytes;
- abnormal pattern expression CD16 vs. CD13 by maturing granulocytes;
- presence of CD34 on maturing myeloid cells or monocytes;
- abnormal pattern expression CD11b vs. HLADR in maturing myeloid cells and monocytes; and
- abnormal pattern expression CD66b vs. CD33.

14.4.1.2 Abnormal Intensity of Antigen Expression in MDS: Abnormal Levels of Antigen Expression Can Be Detected in MDS

Examples of abnormal antigen intensities detected by flow cytometric immunophenotypic analysis in MDS are as follows:[29-31,35,36]

- decreased CD71 in erythroblasts;
- decreased CD10 expression by granulocytes;
- decreased CD16 expression by granulocytes;
- decreased CD45 expression by granulocytes;
- decreased percent CD66 bright cells;
- abnormally bright CD2 expression by myeloid cells;
- high CD36 expression on CD45 dim blasts; and
- increased CD45 on erythroid precursors.

14.4.1.3 Abnormal Antigen Profile in MDS

The absence of normal antigens or the presence of abnormal antigens have been detected in MDS. Examples of abnormal antigen profiles detected by flow cytometric immunophenotypic analysis in MDS are as follows:[29,30,36,37]

- aberrant expression of CD56 on myeloid or monocytic cells;
- CD64 negative granulocytes;
- myeloid lineage expressing lymphoid antigens such as CD22 or CD7;
- lack of CD33 on maturing myeloid cells or monocytes; and
- lack of CD13, CD33, or CD14 on monocytes.

14.4.2 Flow Cytometric Immunophenotypic Prognostic Indicators in MDS

There are data indicating that specific immunophenotypic profiles, as well as a high number of immunophenotypic abnormalities, are associated with a poor International Prognosis Scoring System Score and Risk Categories. Furthermore, high numbers of flow cytometric immunophenotypic abnormalities are associated with posttransplantation relapse and poor overall survival. Flow cytometric immunophenotypic analysis provides prognostic information that can be independent of the International Prognosis Scoring System in predicting relapse and survival.[30]

14.5 Data Reporting

Data reporting should contain not only a description of results, but also convey an appropriate message to the physicians who directly or indirectly manage the patient from whom the sample was obtained. The final report should include the following information.

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14.5.1 Patient Information

Demographics, referring physician and institution, patient history and clinical findings, prior therapy (if relevant to the results), presumptive diagnosis, and reason for FCM analysis should be included. Previous FCM studies, and other laboratory data should also be included, if applicable.

14.5.2 Sample Information

Identification number, source and type, and date of its collection and arrival at the laboratory should be included. A description of the sample, as well as of other materials received, and other procedures should be mentioned, if applicable.

14.5.3 Sample Preparation and Staining

A brief description of cell isolation and purification, cell yield and viability, and staining should be indicated. A list of antibodies used should also be included. The laboratory should internally keep information on instrument QC, date of the analysis, file list and technologist's identification, antibody trade names, clones, and lot numbers.

14.5.4 Results

If abnormal (neoplastic) cells are detected, include a descriptive summary of their phenotype, including cell size, granularity (if applicable), and immunophenotype. The latter should mention also fluorescence intensity for relevant antigens. If contributory, representative graphs illustrating relevant data may be helpful in some cases. The analytical techniques employed to identify abnormal cells and the phenotypic characteristics of abnormal cells can be adequately described in a text format (e.g., B-cell or large cell gating for kappa and lambda expression), without the requirement for numerical results. Reporting of numerical values (i.e., percentage positive) for each antibody in a tabular form is not recommended to indicate the presence of abnormal cells. This approach cannot describe in detail the phenotype of the abnormal cells, and limits the ability of the recipient of the report to interpret results.

When relevant, include the fraction of abnormal (neoplastic) cells present in the sample. Also, the CD4:CD8 T-cell ratio or the kappa:lambda B-cell ratio may be included, if pertinent. When appropriate (e.g., blood), normal ranges should be included. If no abnormal cells are detected, differential counts of the normal elements identified may be provided, if informative.

14.6 Regulatory Statement

Regulation of clinical flow cytometric immunophenotyping of hematolymphoid malignancies differs from country to country. In some situations, regulatory agencies require embedding specific statements within the final data report. An example is the requirement in the United States that reports contain a statement about test approval when using analyte-specific reagents. Since the use of antibodies for flow cytometric immunophenotypic analysis of hematolymphoid malignancies is not approved by the Food and Drug Administration (FDA), they are considered as analyte-specific reagents or "home brew," and must be validated by the laboratory performing the testing. An example of an appropriate analyte-specific reagent statement is as follows:

"These tests were developed and their performance characteristics determined by the Flow Cytometry Laboratory. They have not been cleared or approved by the US FDA, nor do they require this approval."

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15 Data Storage

The possibility of patients or their legal representatives contesting diagnostic implications derived in part from flow cytometric phenotypic analysis makes it incumbent on the laboratory to demonstrate and verify the process used in arriving at the reported test results. Both the data stored and the methods used to obtain the data must be thoroughly documented.

15.1 Information Stored

Patient data files should include all parameters used to obtain test results. These data include:

- forward light scatter;
- 90^{o} light scatter (side light scatter [SSC]); and
- all fluorescence parameters.

The variability observed in LS patterns in patient samples requires that data be stored in list-mode (cell-by-cell) form because gating may not be assignable *a priori*. List-mode data storage will not offset the effect of improper instrument settings or improper sample preparation. Instrument setup and sample preparation are information data sets that must be stored with the list-mode data collected by the instrument (see Appendix A and Section 9).

Quality assurance/QC data records (files) should include all parameters, analysis regions, and analytical results used to verify system (instrument and method) performance.

15.2 Types of Data Storage

15.2.1 Paper Hard Copy

Because data storage is recommended as list mode, paper hard copy is reserved for data presentation. All data plots and analysis regions used in arriving at the reported test results (e.g., gating regions and analysis boundaries), whether obtained from the list mode or from real-time data, must be recorded on the hard copy of the relevant data plot. A unique specimen identifier should also be recorded with each data plot and a cross reference to the originating list-mode data file.

15.2.2 Archival Files

A variety of data storage media may be used, depending on the particular systems available in the laboratory.

If data storage software does not provide for storage of relevant gate information and analysis regions, these data must be recorded as paper hard copy (e.g., amorphous gating regions) or with the test record (e.g., orthogonal gates and analysis boundaries). If the data presentation software does not permit the display of all cell regions in the parameters on which gates are used, separate, ungated data must be made available to permit the clinician to ascertain the validity of the gated portion, with respect to the entire data set.

15.2.3 Duration of Storage

Patient data should be kept for a minimum of two years, or as required by local or national guidelines, whichever is longer. After that, it is the responsibility of the laboratory director to determine whether to save or discard the data. Quality assurance/QC data should be kept for a minimum of two years.

Data may be stored for as long thereafter as the laboratory director considers necessary, provided storage space is available.

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Appendix A. Instrument Setup and Quality Control of Instrument Performance

A1. Introduction

The verification of the reproducibility of instrument performance is an essential element of the daily quality assurance in flow cytometry. Instrument performance must be monitored under the same conditions as those used to run test samples. QC of instrument performance, therefore, involves two procedures:[1]

- **Initial instrument setup**—identification of appropriate conditions for analysis of test samples and establishment of acceptance values for materials used in daily instrument monitoring. This procedure is carried out at relatively large intervals (e.g., once every six months) by qualified service personnel and includes examination of the efficiency and performance of the laser tube, optical filters, log and linear amplifiers, and photomultiplier tubes (PMT) of all types of flow cytometers. These procedures also include calibration of the optical alignment of flow cytometers equipped with a cuvette to ensure that the brightest and tightest peaks are produced in all parameters. Most clinically used flow cytometers are of the "stream-in-cuvette" type. However, optical alignment of the larger flow cytometers with sorting facilities, which are equipped with a nozzle ("stream-in-air" type), must be calibrated daily by the instrument operators, due to its relative instability.

- **(Daily) monitoring of instrument performance**—This group of procedures consists of frequent monitoring of instrument performance by the operators to identify both immediate and potential problems (i.e., with each "cold start" of the instrument). After having established the proper instrument settings, channels and coefficients of variation (CV) for important parameters using suitable reference microbeads are recorded (see below), and the results of the day are then compared with established tolerance limits. Trends and variations can be noted with the use of Levy-Jennings style charts that allow visual inspection of longitudinal data for monitoring instrument performance for precision and trends. If any value falls outside the tolerance limits, monitoring should be repeated and instrument settings adjusted if problems persist.

  Many of the issues involved in instrument QC for immunophenotypic analysis of hematologic malignancies are similar to those encountered in analysis of non-neoplastic WBC. However, the issues of sensitivity and color compensation take on particular importance. Neoplastic WBC frequently exhibit aberrant intensities of antigen expression and/or aberrant patterns of antigen coexpression. If instrument sensitivity is inadequate, dim antigen expression may not be detected. If color compensation is excessive, a population expressing one antigen may be incorrectly considered to lack another. Therefore, it is particularly important to validate instrument performance by using samples with characteristics as similar as possible to those of the test samples.

  Table A1 summarizes the various tasks involved in instrument quality assurance and the general properties of samples that are necessary in performing each task. For each quality assurance (QA) function, the corresponding section in this appendix is specified.

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Appendix A. (Continued)

Table A1. Quality Assurance (QA) Overview

QA Function	Why	When	What (Sample Properties)
Initial Instrument Setup	**Optimize Analysis Conditions**	**New Test or Instrument Conditions**	**Various (see below)**
Optical alignment (A2.1)	Maximize intensity, minimize variability	New instrument, major maintenance or repair	Homogeneous signal in all channels used for specimen analysis; long-term stability reference beads
Position of the "window of analysis" in "sample space" (A2.2)	Adjust the amplifier gain or PMT settings for each parameter, such that the routine specimens for that application appear in the range of histogram channels of these parameters.	New instrument, major maintenance or repair Introduction of test; after any significant changes in specimen preparation or staining protocol	"Normal" specimen prepared using the laboratory's standard test protocol or reference beads with predefined acceptance values established under initial optimal test-specific instrument settings
Setting electronic compensation for spectral overlap (A2.3)	Verify that after labeling of two or more different fluorochromes, multiple-labeled cells can be accurately distinguished from single-labeled cells.	New instrument, major maintenance or repair Each time FL PMT settings are changed	Cells labeled with reagents giving dim-to-bright intensities conjugated to all relevant fluorochromes (e.g., CD8-FITC, CD8-PE) or fluorochrome-conjugated or labeled beads with acceptance values confirmed by labeled cells and with intensities and spectral characteristics similar to bright antibody-positive cells
Linearity validation (A3)	Verify linear detector response	New instrument, major maintenance or repair	Series of beads with at least four different predefined levels of FI, including nonfluorescent beads
Instrument performance monitoring	**Ensure acceptable performance**	**Whenever samples are run**	**Stable; having established acceptance values**
Monitor intensity performance and fluorescence resolution (A4)	Verify reproducible intensity measurements and acceptable sensitivity	Whenever samples are run or instrument malfunction is suspected	Reference beads with predefined acceptance values established under optimal test-specific instrument settings
Calibration of the instrument's response to fluorescent signals (A3)	Standardize measured fluorescence intensity (FI)	Whenever quantitative flow cytometry (QFCM) is performed	Bead(s) or cell(s) with predefined fluorescence intensity (intensities)

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Appendix A. (Continued)

A2. Initial Instrument Setup

A2.1 Optimization of Optical Alignment

Proper alignment of the optical components of the flow cytometer (e.g., laser, flow cell, focusing lenses, collecting lenses, photodetectors) should be established using the manufacturer's recommended alignment materials and procedures. Alignment particles are typically uniform latex particles incorporating a fluorescent dye; other materials may also be recommended by the manufacturer.

The goal in optimizing alignment is to simultaneously achieve maximum signal intensity (i.e., maximum mean channel number) and minimum signal variability (i.e., minimum coefficient of variation [CV]) for all parameters that will be used to analyze test specimens, including forward and sideward light scatter.

For instruments with fixed optical systems, such as most current bench-top flow cytometers where alignment is not carried out daily by the user, alignment should be verified using manufacturer-recommended particles to determine whether instrument performance meets specifications.

In some cases, manufacturers provide expected values for their particles when used, for example, with specific instruments, instrument settings, and filters. In other cases, the laboratory must determine optimum settings for its own instrument-alignment particle combination and establish its own expected values.

A2.2 Positioning of the "Window of Analysis" in "Sample Space"

Dependent on the application, the amplifier gain or PMT settings for each parameter require adjustment, such that the routine specimens for that application appear in the range of histogram channels of these parameters (i.e., the "window of analysis"). Changing the amplifier gain or PMT settings repositions this window in "sample space" (i.e., the fluorescent and light scatter characteristics of the sample that are independent of the instrument). For positioning of the fluorescence (FL) windows of analysis, it is recommended that, for each type of assay, unstained cells are placed in the first of four log decades, above the level of instrument noise. Depending on the fluorochrome, autofluorescence of unstained cells may be greater than that of unstained beads at the recommended PMT setting.

This approach has the following advantages:

- The observation that unstained beads often fall in a lower range of channels than unstained cells ensures that the instrument's noise does not interfere with the result of the immunophenotyping assay.

- The positioning of single-stained and unstained cells slightly away from the axes of the bivariate FL allows appropriate adjustment of electronic compensation for spectral overlap.

A2.3 Setting Electronic Compensation for Spectral Overlap ("Color Compensation")

Compensation is the process of correcting for spectral overlap of one fluorochrome into the filter window used to monitor another fluorochrome (see Figure A1). In most instruments used clinically, this correction is done by adjusting the electronic compensation circuits on the flow cytometer to place populations not expected to be dual-positive for two fluorochromes into orthogonal fluorescence quadrants, with no overlap into the double-positive quadrant. At the same time, avoiding overcompensation is essential because overcompensation may cause incorrect classification of dual-positive cells as single positive. The procedure may be performed manually; alternatively, software may perform the spectral

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Appendix A. (Continued)

compensation automatically (see below). Spectral overlap of fluorochromes must be corrected for to ensure the accurate enumeration of cells labeled with multiple fluorochromes. Accurate compensation is accomplished by staining a control blood sample containing mutually exclusive populations of cells carrying the same fluorochrome as used in the experimental samples, and by processing this control sample in an identical way to the experimental samples. (The best method is to singly stain cells using each of the antibodies in the experiment. This way, the laboratorian ensures that the spectral characteristics for each antibody are correctly represented.) In this respect, the use of blank and externally labeled beads may be inferior to the use of cells, because the spectral characteristics of fluorochromes directly bound to beads are slightly different from those of fluorochromes bound to antibodies. Especially on instruments using analog log amplification, setting color compensation using the FL signal of labeled beads relative to that of blank beads results in slight overcompensation, because blank beads have dimmer FL signals than unstained cells due to the autofluorescence of the latter. If standardization or calibration particles (microbeads) are used to set compensation, appropriate color compensation can be confirmed by using lymphocytes labeled with FITC- and PE-labeled monoclonal antibodies and a third-color- or fourth-color-labeled monoclonal antibody for three-color or four-color panels, respectively.

The spectral characteristics of most fluorochromes (i.e., FITC, PE, PerCP, APC) will be similar when conjugated to different antibodies. Exceptions to this rule are the tandem fluorochromes (see below). The electronic correction of spectral overlap is a function of the PMT settings involved. Therefore, each time the settings of an FL PMT are changed, compensation settings must be verified and corrected. In contrast, changing FSC photodiode or SSC PMT settings does not affect compensation.

In the setting of multicolor analyses, visualization of compensated data may often result in unexpected distributions—mainly the appearance of large numbers of events on the axis, but also the inability to reduce the range of compensated data to "autofluorescence" levels. In such situations, it is impossible to achieve appropriate color compensation using standard visual graphical interfaces (i.e., bivariate histograms using log scales); manual adjustments of compensation will always result in overcompensation. Therefore, computer-assisted adjustment of compensation is required, as well as careful gating and experimental design to discriminate between positive and negative events. At a minimum, such software should provide median statistics or a graphical representation of the median as a function of intensity of positive and negative populations during any user-controlled adjustment. Alternatively, all adjustments are made automatically without user intervention.[2,3]

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Appendix A. (Continued)

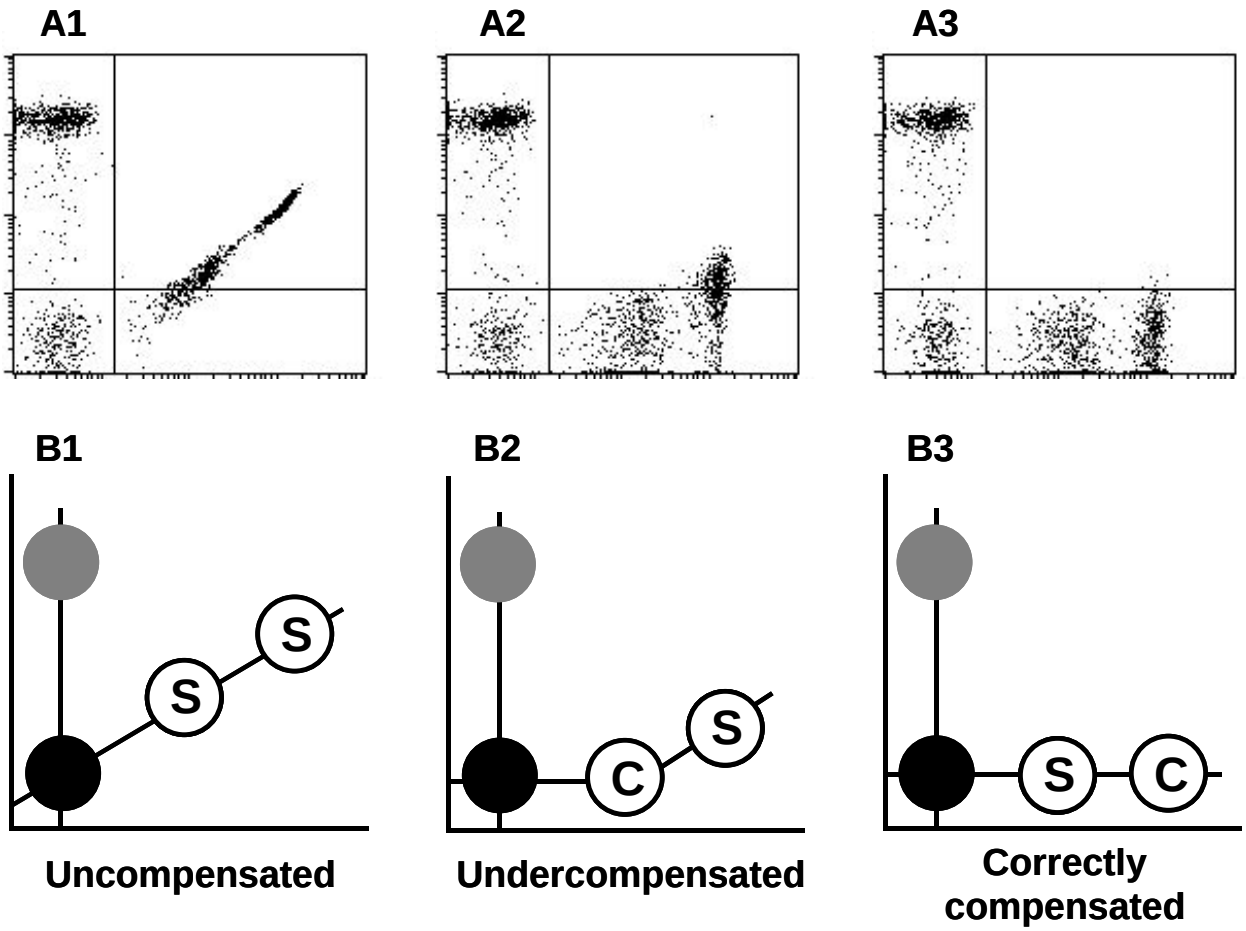

Figure A1. Histograms A1 to A3 and their respective schematic depictions, B1 to B3, illustrate uncompensated, undercompensated, and correctly compensated two-color displays, respectively. The open circles labeled "S" represent the cell population for which accurate color compensation is desired. The open circles labeled "C" represent the compensation controls used to adjust color compensation settings. If the intensity of the control does not exceed the fluorescence intensity of the sample analyzed, that sample will be undercompensated (A2, B2). If the intensity of the control exceeds the fluorescence intensity of the sample analyzed, the sample is less likely undercompensated (A3, B3). **NOTE:** In panels A3 and B3, the median fluorescence (y-axis) of the unstained, compensated particles and stained cells will be approximately equal.

With the expanding parameter capability in flow cytometry, newly developed fluorochromes now include the so-called tandem dyes, whose design is based on the principle of fluorescence resonant energy transfer (FRET). In FRET, the donor dye absorbs energy from the illuminating source and transfers that energy to a partner or acceptor dye without emitting photons. The partner dye then emits photons at its own characteristic wavelength, permitting excitation of several colors from a single laser. Nevertheless, for some conjugates, FRET is incomplete, and compensation must be used to suppress the effect of photons "leaking" at the donor dye's usual emission wavelength. "Good" tandem dyes require minimal compensation (1 to 5%) of the donor dye parameter, but some other tandems may require ≥ 10% compensation, jeopardizing the detection of weakly expressed antigens by masking fluorescence. This problem is aggravated by the fact that quite often, the very same fluorochrome as is the donor dye in the tandem (i.e., phycoerythrin [PE]) is selected for measurement of dimly expressed antigens because of the

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Appendix A. (Continued)

brightness of PE. To avoid this "loss of sensitivity," staining panels should be constructed, selecting those antigens whose expressions are mutually exclusive for the single-color donor dye and the tandems, respectively. Figure A2 illustrates five commonly used fluorochromes with measurable "leakage" into other fluorochromes. The significant leakage of the tandem fluorochrome PC7 (PE-Cy7) into the emission wavelength of PE should be noted. Importantly, spectral overlap is not unique to tandem dyes; in particular, FITC has significant overlap into PE, and PE into ECD (PE/Texas Red tandem fluorochrome). Fluorochrome/antibody combinations should always take these overlaps into account when designing panels.

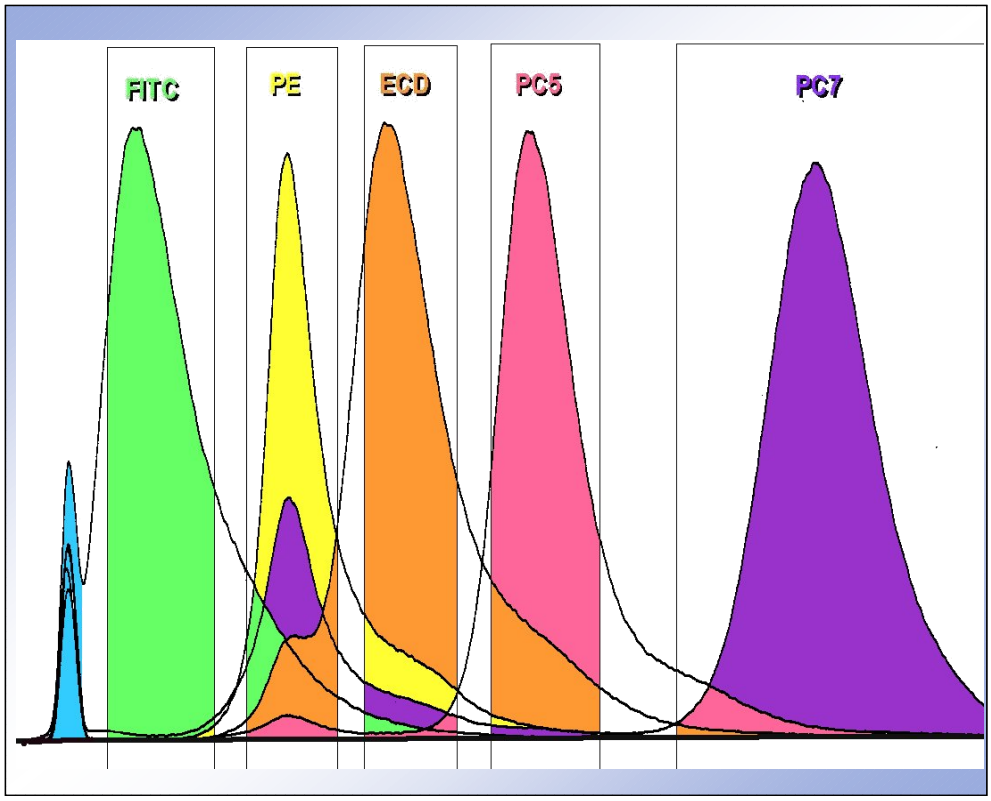

Figure A2. Spectrum of Wavelength Emission of Various Fluorochromes. To simplify the display, the color intensities of the various fluorochromes have been normalized. PC5=PE-Cy5. PC7=PE-Cy7.

A3. Linearity Validation and Calibration of the Instrument's Response to FL Signals

Since fluorescence intensity is used to distinguish "positive" from "negative" cells in immunophenotyping assays, it is important to ensure reproducibility and stability of FL intensity assessments over time. This QA procedure is done by calibrating the flow cytometer using a series of beads with at least four different predefined levels of FL intensity, including nonfluorescent beads.[4,5] In the most current edition of CLSI/NCCLS document I/LA24—*Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity,* the basic principles and laboratory practices involved in quantitative flow cytometry (QFCM) are outlined. This guideline allows the generation of a linear calibration curve for each fluorescence detector and for a given flow cytometric assay. In this context, it is important to differentiate between a linear detector response and the linear range of a specific assay. However, the principles are applicable to both situations.[6,7]

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Appendix A. (Continued)

QFCM is the measurement of FL intensity in terms of relative fluorescence intensities (RFI) or molecular equivalents of soluble fluorochrome (MESF).[7] For each fluorochrome, a calibration standard is constructed to obtain a standardized determination of FL intensity. A reference method is described for constructing an FI calibration curve and evaluation of instrument performance parameters.[7] The proper construction and interpretation of a calibration curve for QFC requires the selection of appropriate standards,[5] as well as the correct transformation of units of FL intensity for reporting results.[7]

There are five recognized approaches for generating a standard calibration regression line. First, the simplest approach consists of internally dyed microspheres that have been assigned relative FL intensity (RFI) values, as these standards do not spectrally match the fluorochromes that are routinely used for QFCM (e.g., FITC, PE, APC). Second—and in contrast to the first approach—MESF microbead standards do spectrally match these fluorochromes. In this context it is relevant that that National Institute of Standards and Technology (NIST) has developed a fluorescein reference solution (SRM 1932) for use in conjunction with the FITC-labeled microsphere reference material RM 8640.[8] Third, a modification of this approach is based on commercially available PE-labeled microsphere calibrators used in conjunction with mAb that have been labeled 1:1 with PE (i.e., 1 PE molecule to 1 mAb molecule). The calibration regression line constructed in this way allows the expression of FL intensity in terms of numbers of mAb molecules bound per cell (ABC).[9-11] Fourth, Giorgi et al combined an RFI-based calibration regression line with a biological calibrator (i.e., $CD4^+$ T-cells). As the number of CD4 molecules per T-cell is very stable (i.e., 50 000 per cell), this characteristic was used to derive the numbers of molecules of interest per cell (such as CD38) from the calibration curve.[12] In a further variation of this method, Davis et al used a known MESF microbead to standardize the measurement of CD64 expression for the diagnosis of sepsis.[13] Fifth, Poncelet et al developed biological calibrators to construct a calibration regression line for indirect immunofluorescence stainings.[14]

Most of these systems allow the user to generate calibration regression lines, and to derive from these lines instrument performance parameters for each FL channel. Five of these parameters have been defined: linearity or average residual percent, slope or coefficient of response, zero channel, maximum channel, and detection threshold (see Table A2). The average absolute percent residual gives the average percent disagreement between the assigned and best-fit dose values for all data points, and is the simplest aggregate parameter of overall linearity. The slope or coefficient of response is a measure of the number of channels per decade on a log scale. Extrapolating the calibration curve to the zero and maximum channels can be used to define a common window of analysis (see above). The use of a consistent window of analysis may facilitate quality assurance in longitudinal and multicenter studies. The detection threshold is complicated both from the standpoint of instrumentation and the biological assay.[15-17]

In summary, instrument performance parameters provide the most objective way to validate instrument performance and compare FL measurements across time, instruments, and platforms. Thus, monitoring of these parameters provides the most comprehensive approach to instrument QC and assurance of FL measurement accuracy.

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Appendix A. (Continued)

Table A2. Instrument Performance Parameters for Measurements of Fluorescence[7]

Average residual percent	
Definition	The absolute average percent that the calibration regression line between a series of calibration beads labeled with different FL signals of predefined intensities, and the instrument's response (in histogram channels) deviates from the actual data points.
Significance	*Indicates:* • the linearity or goodness of fit of the calibration line; and • the confidence of the instrument's response across the FL intensity range.
Tolerance zone	*(arbitrary)* < 5%
Detection threshold	
Definition	Value, in arbitrary units of FL intensity, of the histogram channel at the right-hand foot of the peak of nonfluorescent standards.
Significance	*Indicates:* the lowest level of fluorochrome that can be detected by the instrument.
Tolerance zone	*Defined by the user*
Coefficient of response	
Definition	The slope of the calibration regression line (see under "Average residual percent") calculated using a linear scale. Indicates a theoretical value for the number of histogram channels per decade on a log scale (e.g., four-log decade and 256-channel resolution: 256/4 = 64.0).
Significance	*Indicates:* • range of sample space covered by the window of analysis; and • deviation from the theoretical value indicates suboptimal instrument performance.
Tolerance zone	*(arbitrary)* (instrument channel resolution ÷ # log decades) ± 5%
Zero channel value	
Definition	Intercept of the calibration line with the axis on which units of FL intensity have been plotted.
Significance	*Indicates:* theoretical number of units of FL intensity corresponding with histogram channel 0; hence, it defines the left-hand boundary of the FL window of analysis.
Tolerance zone	*Defined by the user*
Maximum channel value	
Definition	Intercept of the calibration line with the axis on which units of FL intensity have been plotted.
Significance	*Indicates:* theoretical number of units of FL intensity corresponding with maximum histogram channel; hence, it defines the right-hand boundary of the FL window of analysis.
Tolerance zone	*Defined by the user*

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Appendix A. (Continued)

A4. Monitoring of Instrument Performance

In order to maintain appropriate instrument settings over time, control or reference materials are measured under test-specific instrument settings, and the values obtained are compared to the acceptance values previously established under conditions of optimal performance. Upon each "cold start" of the instrument, appropriate application-specific instrument settings must be obtained.

Two different strategies exist for this purpose:[1]

Strategy 1: Reestablish identical target channels (see Figure A3-1)

According to this strategy, daily adjustment of PMT settings of all FL parameters is performed to maintain a constant positioning of the window of analysis in sample space. For each FL parameter, reference beads are placed in identical target channels between runs. The daily adjustment of PMT settings implies that compensation settings must also be adjusted daily. The major manufacturers of flow cytometers have developed reagents and automated software for this purpose.

Strategy 2: Meeting "tolerance zones" (see Figure A3-2)

The appropriate positioning of the FL windows of analysis in sample space is monitored by verifying, using reference beads, whether or not measurement of reference beads after each "cold start" fall within predefined "tolerance zones." For this purpose, the mean FL channels of the reference beads are plotted in Levey-Jennings charts. Adjustment of PMT settings and color compensation is only necessary if beads repeatedly fall outside these tolerance zones, which may be evaluated on the basis of Westgard's rules.[18] Once an instrument is serviced, tolerance zones will most likely require redefinition.

Comparison of both strategies

Both approaches (i.e., reestablishing identical FL target channels, and meeting predefined FL tolerance zones over time) ensure that data collected over long periods of time will be meaningfully comparable, even if the instrument's performance was variable during that period, because the instrument's responses to FL signals have been adjusted to perform relative to an independent benchmark. The advantage of Strategy 1 is its user-friendliness, thanks to the use of instrument-specific software. However, without this software, the approach would be very labor-intensive. Therefore, the manufacturer-defined procedures should be strictly followed. The advantages of Strategy 2 are twofold: (1) universal applicability (some instrument-specific systems based on Strategy 1 do not accommodate the use of specific fluorochromes); and (2) labor saving when multiple application-specific instrument settings have been verified at the same time (e.g., when multiple tandem conjugates are used, or when multiple sample preparation methods require specific instrument settings). If one of these settings is appropriate, it implies that the remaining settings are also adequate at that time point.

NOTE: In spite of proper alignment, calibration, and compensation control during system setup and QC, the quality of data generated is not guaranteed. Momentary fluidics variations, sample aggregations, or even occasional air bubbles passing through the flow cell can cause disturbances in the measured parameters. Looking at ungated data for shifts in light scatter characteristics helps to detect this problem before further analysis.

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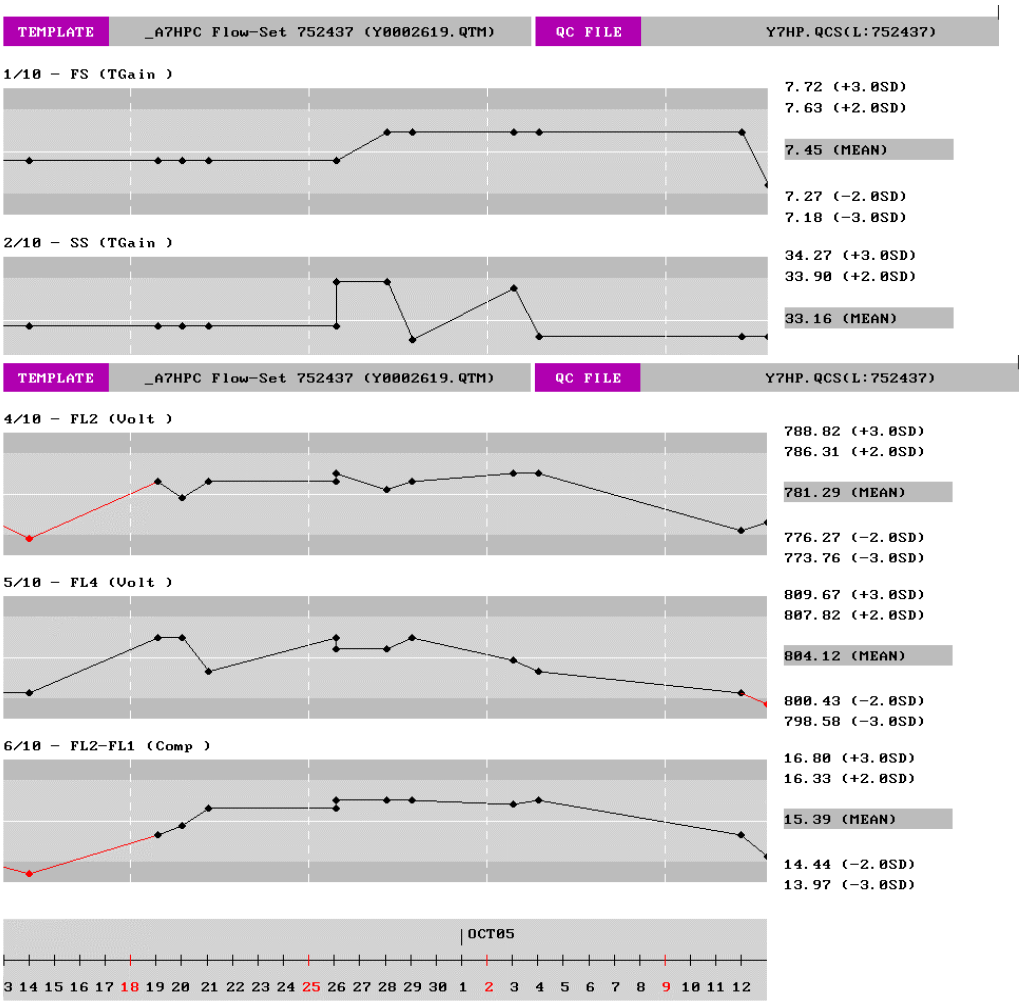
Appendix A. (Continued)



Figures A3-1 to A3-3. Illustration of Two Strategies for Instrument Performance Monitoring

Figure A3-1. Strategy 1: detector gain or high voltage required to reproduce predefined mean channel number of a fluorescent setup bead for forward scatter (FS), side scatter (SC), FL2, and FL4. Compensation (FL2 to FL1) is shown in the lowest panel. The area with light-gray background indicates the "narrow-margin" tolerance zone (i.e., mean ±2 SD), while the area with dark-gray background indicates the extension to a "wide-margin" tolerance zone (i.e., mean ±3 SD).

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Appendix A. (Continued)

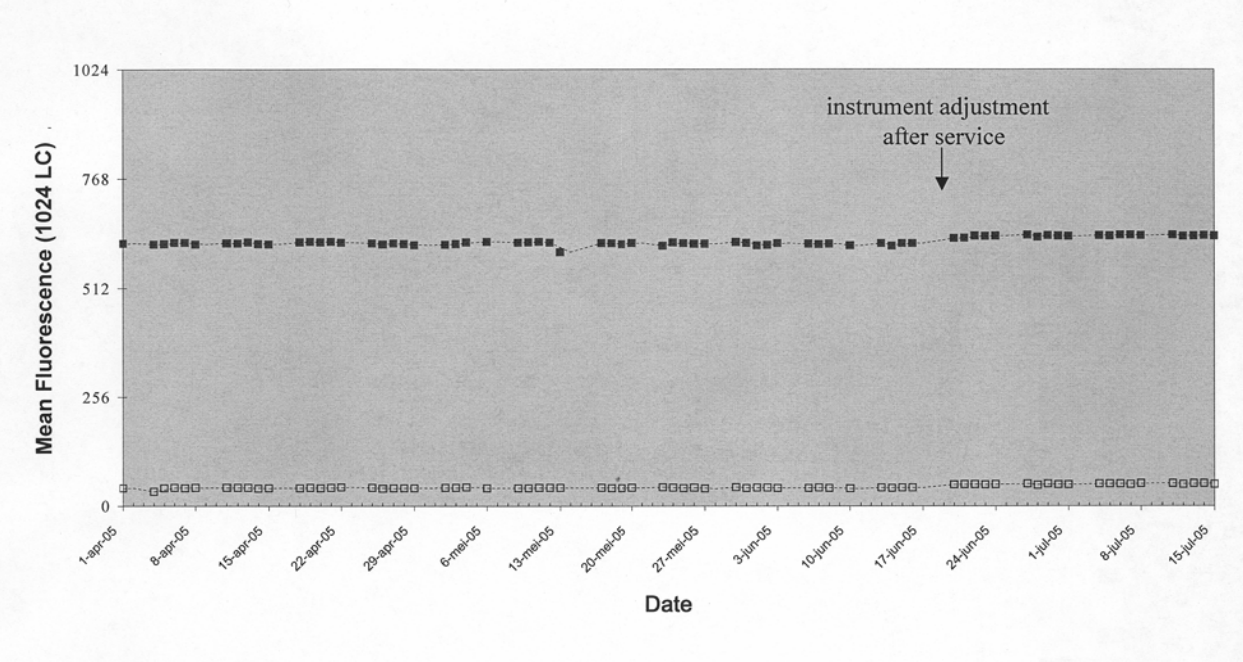

Figure A3-2. Strategy 2: mean fluorescence intensity of a blank bead (open squares) and fluorescent reference beads (closed squares) for an FL channel monitored under constant instrument conditions (i.e., constant FL detector high voltage/gain settings, compensation settings). The instrument was serviced on 19 June 2005, after which its settings were changed.

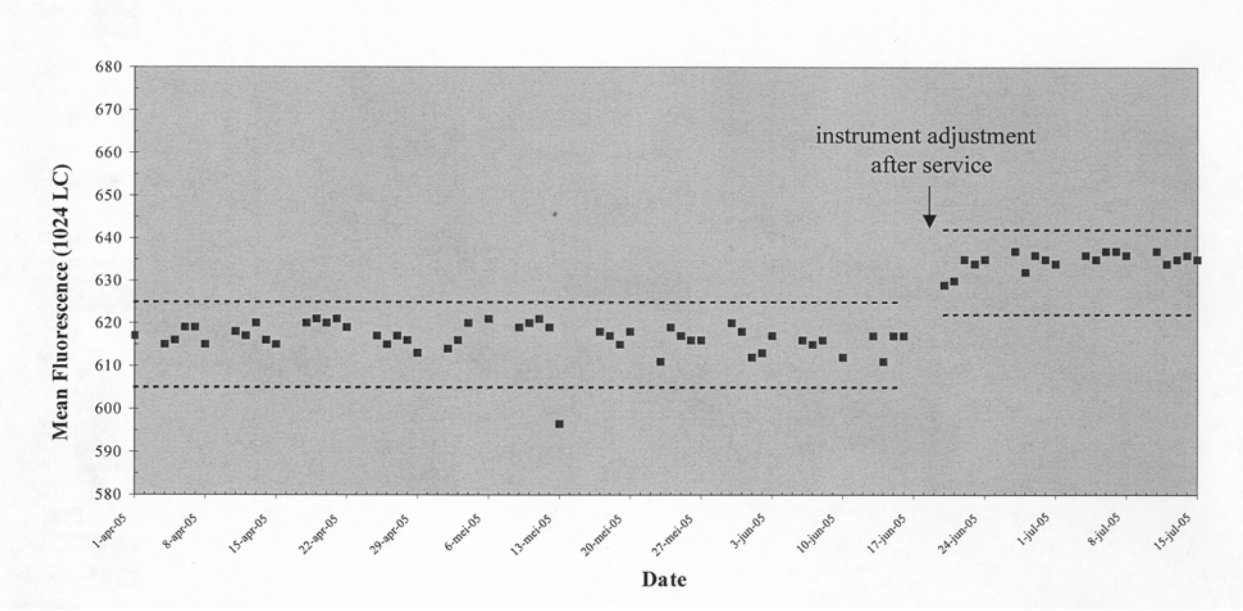

Figure A3-3. Magnification of the data on the fluorescent reference beads in Figure A3-2. The dashed lines indicate the established tolerance zones (mean ±10 channels; 1024-channel linear histogram scale). The out-of-range value obtained on 13 May 2005 was related to temporary blockage of the instrument's flow cell, which was solved by simple rinsing.

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Summary of Delegate Comments and Subcommittee Responses

H43-A2: *Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition*

General

1. When is flow cytometry NOT indicated for the evaluation of liquid and solid tissue specimens for leukemias and lymphomas?

- **Within the "Scope," it is stated that it is beyond the scope of this document to describe completely when flow cytometric analysis of leukemia or lymphoma is appropriate. Likewise, it is beyond the scope of this document to describe completely when flow cytometric analysis of leukemia or lymphoma is not appropriate. These decisions are based upon medical knowledge, specimen size as well as condition, ancillary tests available in the institution and available patient history, signs, and symptoms. The following comment was included to further define the scope of the document: "Because this document is intended primarily for laboratory workers in flow cytometry, it cannot describe all the possible clinical situations in which flow cytometric analysis of leukemia or lymphoma is or is not appropriate."**

2. Flow immunophenotyping still remains an ancillary technique, albeit one that is often legitimately applied to an ever widening number of leukemias and lymphomas. However, the statement in Section 1, "Therefore, immunophenotypic analysis of hematolymphoid neoplasia is crucial for the accurate diagnosis and classification of these complex malignancies" should be modified to indicate that (a) immunophenotyping is not always required (e.g., for follicular lymphoma on biopsy), and (b) alternative phenotyping via immunohistochemistry may suffice.

- **While one might argue that immunophenotypic analysis is not required for diagnosis of absolutely all hematolymphoid malignancies, it is prudent to use immunophenotypic analysis for the diagnosis of hematolymphoid malignancies. For this reason, immunophenotype plays an important role in the WHO *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.* As mentioned in the introduction, "Some diseases have a virtually specific immunophenotype, such that one would hesitate to make the diagnosis in the absence of the immunophenotype" (Jaffe ES, Harris NL, Stein H, Vardiman JW, eds. *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon: IARC Press; 2001:12). It is the experience of the subcommittee that hematopathologists rely heavily on immunophenotypic analysis when rendering their final diagnosis and thus this is a crucial ancillary test. We do not specify whether immunophenotyping should be accomplished by flow cytometry or immunoperoxidase studies of paraffin sections, as choice of test depends upon resources available and expertise in interpretation. Recommendations upon choice of methodology for immunophenotyping are beyond the scope of this document. The current document is intended primarily for laboratory workers in flow cytometry and thus only describes guidelines for flow cytometric immunophenotyping.**

3. Morphologic triage and waiting 24 to 48 hours for such is a legitimate approach to decide whether to order or not order immunophenotyping, and, if so, whether or not flow or immunohistochemistry is the best approach. This needs to be clearly stated, ideally in a section related to this (e.g., Section 4.1). Flow samples, particularly liquid heme (blood, marrow), can sit for 24 to 48 hours without significant loss of antigenicity. Further, crude cell suspensions of tissue can easily maintain antigenicity for 24 hours.

- **Section 4.1, Goals discusses the goals of flow cytometry, not triage of specimens.**

  As specimen age is a critical variable in flow cytometric analysis, recommendations are given as to acceptable cutoffs in specimen age based upon specimen type and other factors, such as anticoagulant (24 to 72 hours). Recommendations are also made for proper storage, based upon specimen source and

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presence of anticoagulant. While, in general, it is true that specimens may be stored before morphological triage, decreasing viability with specimen age is not always predictable and is a particular problem in cases of tumors with high proliferation rate (e.g., Burkitt lymphoma) or in patients where chemotherapy and/or radiation treatments were given. Furthermore, some antigens are more labile than others (e.g., CD 138 and CD 16) and are preferentially affected with increasing specimen age, leading to potential false negativity. Therefore, it is recommended that specimens be processed as expeditiously as possible and that the possible loss of labile antigens be noted in older specimens. Given these qualifying statements, the document is not per se in conflict with the above comment. The document does comment that in shipped specimens, it is not advisable to hold fresh tissue for flow cytometric analysis until after histologic sections are ready, as this *additional* delay could adversely affect the viability of the sample. As always, laboratories should demonstrate that, in their specific application, stored specimens give equivalent results to freshly processed ones in accordance with acceptable medical practices.

Section 6, Safety

4. Safety refers to universal precautions when Section 3, Standard Precautions states the document will follow standard precautions.

- **The document has been edited to contain reference to standard precautions only.**

Sections 7.1.1, Requirements and 9.1.5, Improper Specimen Labeling

5. Number of unique identifiers: Most regulations require two unique identifiers (e.g., name and medical record number or birth date). In these sections, only one unique ID is mentioned.

- **This document is intended to serve as an international laboratory guideline and as such, the number of unique identifiers was kept at the minimum. Section 7.1.1 has been modified to read, "All samples submitted for testing must be labeled immediately at the time of specimen collection with at least one unique patient identifier (in compliance with local regulations) and date and time of collection."**

Section 9.2.5, Processing Lymph Node or Extranodal Tissue Samples

6. Processing for lymph node samples: After saving adequate amount of tissue for routine histologic exam, it is better practice to save a piece of tissue via snap freezing and then, and only then, preparing any remaining sample for flow. Going directly to disaggregation for flow cytometry risks loss of neoplastic cells (e.g., large cell lymphoma). Immunophenotyping can still be done in most instances via paraffin section immunohistochemistry, and molecular and FISH studies can be done both on the snap frozen and paraffin embedded material.

- **Section 9 describes how to process lymph node or extranodal tissue for flow cytometric analysis and is not intended as a guideline for tissue allocation. Recommendations upon choice of methodology for immunophenotyping are beyond the scope of this document. The current document is intended primarily for laboratory workers in flow cytometry and thus only describes guidelines for flow cytometric immunophenotyping.**

Section 9.4, Evaluation of Sample Viability

7. The need for defining and reporting the percent of viable cells is unclear. As a corollary, there really is no significant body of literature that can show labs that routinely do and report viabilities yield better flow analysis or have superior patient outcomes. It would be better to simply indicate that the laboratory should have a policy on when to do viability testing.

- **Although fresh blood and fresh marrow don't necessarily require viability determinations, it is vital that viability be checked on cells obtained from tissue specimens. Viability can be quite low in these cases and viability dramatically affects the quality of flow data. For this reason, the College of American Pathologists requires viability measurements be performed on all cell suspensions from tissue.**

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The reason that viability testing is important is that nonviable cells may nonspecifically bind to many antibodies and interfere with accurate immunophenotyping. For this reason, the document indicates the need for viability determinations whenever there is a high probability that poor viability may be encountered. As it is, the assessment of viability and the reporting of low viability and how it may impact the interpretation of results is important, not the actual percent of viable cells. The first sentence of Section 9.4.2 stating that percent of viability should be reported has been deleted.

Section 10.2, Optimization of Staining Protocol

8. Ficoll purification is a legitimate approach to processing flow samples, particularly solid tissue samples. The statement is largely hypothetical—there again is simply no literature that can show that labs using ficoll purification have any greater yield of false-negative flow results or the patients have clinically inferior outcomes.

- **The US-Canadian Consensus Recommendations on the Immunophenotypic Analysis of Hematologic Neoplasia (*Cytometry Communications in Clinical Cytometry.* 1997;30:2) clearly states that "the preferred method of RBC removal is lysis…". Density gradient separation can result in the selective loss of important cell populations (*J Immunol Meth.* 1983;61:259-260, *J Immunol Meth.* 1987;98:53-56) and therefore lysis is preferred. The document has been revised to indicate that this is a preference, not an absolute requirement. The paragraph on RBC removal has been revised as follows: "In red-cell-containing samples, RBC lysis is the preferred method of RBC removal. Neoplastic cells have greater heterogeneity in density than normal cell populations, and density gradient purification may result in specific loss of abnormal cells."**

Section 14.1, Introduction

9. Last sentence – It is not clear why an appropriately trained individual cannot interpret flow data if he/she cannot directly analyze list mode files. As long as the individual sees the dot plots and has the ability to direct the remote flow lab to reanalyze a certain population (via fax or e-mail of dot plots and the use of a phone), there is not need to have to have the list mode files at the site of the final interpreter, and/or require the final interpreter to analyze the data on a PC. This potentially could be seen as an attempt by centralized flow facilities to restrain "trade" or "competition" by other competent interpreters (e.g., referring pathologists).

- **Diagnostic flow cytometry is generally performed with sample processing, flow cytometric analysis, data interpretation, and interpretive reporting all performed at a single laboratory site. However, there is a subset of pathologists who send specimens to reference laboratories where the sample processing and flow cytometric analysis are performed. The resultant data are then transmitted via various means to the pathologist who independently performs the data interpretation and generates a report to the clinician at the local institution who submitted the specimen for diagnostic evaluation. This practice of so-called technical-only flow cytometry by a reference laboratory is a controversial area within the clinical flow cytometry community for several reasons. Interpretation of flow cytometric data requires an intimate knowledge of normal flow cytometric immunophenotypic patterns so that abnormal cells and technical artifacts can be detected. A pathologist who is well trained in flow cytometric immunophenotypic analysis can competently interpret data generated at an outside reference laboratory. However, data analysis and interpretation in flow cytometric immunophenotyping is complex and should involve the assessment of all cells, not just a subset chosen by a technologist and represented in dot plots. Simply looking at the dot plots of cells in a gate chosen by a technologist is similar to only examining pictures of cells chosen by the histotechnologist who prepares surgical pathology slides. The pathologist is responsible for interpretation of all data acquired and medical knowledge is frequently required to choose the appropriate parameters to study. Furthermore, one cannot ascertain with certainty by examining a printed page what limits on parameters were set by the technologist. For these reasons, we believe that the reference laboratories should make the list mode data available and that the individual interpreting the immunophenotypic data should receive training in how to directly review list mode files.**

Section 14.2.2.1, Precursor B-cell Acute Lymphoblastic Leukemia

10. pB-ALL profile: you may wish to include that a substantial minority is CD45 negative.

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- **At the end of the first paragraph in this section, which ended: "…subset of CD20- cells," the text has been revised by adding the following new sentence: "CD45 fluorescence intensity is highly variable, and in a substantial minority of cases, CD45 may be negative or undetectable in some, or even all of the blasts."**

Sections 14.2.2.5, Minimal Residual Disease Analysis in Acute Leukemia and 14.3.4, Minimal Disease Detection in Mature Lymphoid Neoplasms

11. Similarly, with minimal residual disease (MRD) for acute leukemias and mature lymphoid neoplasias, there currently are no nationally recognized standards that define for clinicians what to do with MRD defined by flow. As a corollary, beyond pure prognostication, there are no defined (nonexperimental) treatments that have been shown to give better outcomes for MRD+ vs. MRD negative cases, particularly for adult acute leukemias and chronic lymphoid neoplasms. In the introduction for both of these sections, these limitations should be noted.

- **In the middle of the first paragraph of Section 14.2.2.5, after the sentence that ends: "…and the detection of such minimal residual disease (MRD) following therapy may be an adverse prognostic factor in certain settings," the text has been revised by adding the following sentence: "Despite the prognostic significance of MRD detection in patients with acute leukemia, it should be recognized that at this time, treatments that impact the natural history of the disease have not yet been established."**

  In Section 14.3.4, the text has been revised as follows: the last sentence of the first paragraph in this section, which began, "Detection of residual disease…" now reads, "Detection of minimal residual disease (MRD) following therapy may provide useful prognostic information, and even herald relapse in patients without clinical or morphologic evidence of overt relapse. Nonetheless, as discussed above with respect to MRD detection in patients with acute leukemia, there are as yet no established treatments that impact the natural history of the disease in patients with mature lymphoid neoplasms."

Section 14.4, Myelodysplastic Syndrome (MDS)

12. Flow for myelodysplasia – It is premature to say unequivocally that "flow cytometric immunophenotypic analysis is useful in diagnosis and as a prognostic indicator in the myelodysplastic syndromes." This should be modified with the caveats that flow immunoanalysis is not currently included in any nationally or internationally accepted criteria for diagnosis or prognosis, and that many clinical hematologists, oncologists, and hematopathologists simply do not know how to apply such data to the treatment and prognostication of their patients. This should be added at the introduction to this section.

- **The literature supports the statement that "flow cytometric immunophenotypic analysis is useful in diagnosis and as a prognostic indicator in the myelodysplastic syndromes." The fact that it is not included in the WHO classification system or that many clinicians do not understand the data does not negate this fact. Flow cytometric immunophenotyping was acknowledged as useful in the diagnosis of non-Hodgkin's lymphomas before immunophenotyping was acknowledged in any international criteria for diagnosis. This document does not suggest that flow cytometric immunophenotyping should be used alone in the diagnosis of myelodysplastic syndrome. The document also acknowledges the difficulty in interpretation of these cases and the extensive knowledge of the normal patterns of antigen expression during myeloid differentiation required. However, in laboratories capable of performing this analysis, flow cytometric immunophenotyping is a useful adjuvant to morphology and cytogenetics in diagnosis. Furthermore, it was demonstrated to predict relapse after bone marrow transplant, making it clinically valuable.**

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NOTES

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The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality management system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any healthcare service's path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Customer Service
Personnel	Process Control	Assessments—External and Internal	Facilities & Safety

H43-A2 addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessments—External and Internal	Process Improvement	Customer Service	Facilities & Safety
				H1 H3	X H3 H20 H26 H42 I/LA24						GP5 H3 M29

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.*

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow, which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

H43-A2 addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Preexamination				**Examination**			**Postexamination**	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
X H3 H42	X H3 H42	X H3 H42	X H3 H18 H42	X H42	X H42	X H42	X H42	

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.*

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Related CLSI/NCCLS Publications*

GP5-A2 **Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002).** Based on US regulations, this document provides guidance on the safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory. While a valuable resource for a wider audience, it is intended for use primarily in the United States.

H1-A5 **Tubes and Additives for Venous Blood Specimen Collection; Approved Standard—Fifth Edition (2003).** This document contains requirements for venous blood collection tubes and additives, including technical descriptions of ethylenediaminetetraacetic acid (EDTA), sodium citrate, and heparin compounds used in blood collection devices.

H3-A5 **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children.

H18-A3 **Procedures for the Handling and Processing of Blood Specimens; Approved Guideline—Third Edition (2004).** This document includes criteria for preparing an optimal serum or plasma sample and for the devices used to process blood specimens.

H20-A2 **Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard—Second Edition (2007).** Discusses automated differential counters and establishes a reference method based on the visual (or manual) differential count for leukocyte differential counting to which an automated or manual test method can be compared, and an experiment to carry out the comparison. Describes procedures for collecting specimens; preparing blood films and requirements for acceptable wedge and spun films; Romanowsky staining; the formed elements; variant leukocyte forms; and a protocol for examining blood films. Details procedures for determining inaccuracy, and within-run and between-run imprecision; procedures for determining sensitivity/specificity/predictive value of flags; and statistical methods for determining inaccuracy and imprecision.

H26-A **Performance Goals for the Internal Quality Control of Multichannel Hematology Analyzers; Approved Standard (1996).** This document addresses performance goals for analytical accuracy and precision for multichannel hematology analyzers; the relationship of these goals to quality control systems and medical decisions; and recommendations for minimum calibrator performance and the detection of measurement errors.

H42-A2 **Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition (2007).** This document provides guidance for the immunophenotypic analysis of non-neoplastic lymphocytes by immunofluorescence-based flow cytometry; sample and instrument quality control; and precautions for acquisition of data from lymphocytes.

I/LA24-A **Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Approved Guideline (2004).** This guideline describes the basic principles, reference materials, and laboratory procedures upon which quantitative fluorescence calibration is based.

M29-A3 **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.

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St. Eustache Hospital (Quebec, Canada)
St. Francis Medical Center (MN)
St. John Hospital and Medical Center (MI)
St. John's Hospital & Health Ctr. (CA)
St. Joseph Medical Center (MD)
St. Joseph Mercy (WI)
St. Joseph Mercy Hospital (MI)
St. Joseph's Hospital (FL)
St. Joseph's Medical Center (CA)
St. Joseph's Regional Medical Center (NJ)
St. Jude Children's Research Hospital (TN)
St. Louis Children's Hospital (MO)
St. Luke's Hospital (PA)
St. Margaret Memorial Hospital (PA)
St. Mary Corwin Regional Medical Center Laboratory (CO)
St. Mary Medical Center (CA)
St. Mary's Health Center (MO)
St Mary's Healthcare (SD)
St. Mary's Hospital (BC, Canada)
St. Mary's Medical Center (IN)
St. Rose Dominican Hospitals (NV)
St. Thomas More Hospital (CO)
San Antonio Community Hospital (TX)
San Francisco General Hospital-University of California San Francisco (CA)
Santa Monica Hospital Med. Ctr. (CA)
Seoul Clinical Laboratories (Korea)
Shands at the University of Florida
Shape Healthcare Clinic (APO, AE)
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South Florida Baptist Hospital (FL)
South Texas Laboratory (TX)
Southern Health Care Network (Australia)

Southwest Nova District Health Authority (Canada)
Specialty Laboratories, Inc. (CA)
Squamish General Hospital (BC, Canada)
Starke Memorial Hospital Laboratory (IN)
State of Connecticut Department of Public Health (CT)
State of Washington Public Health Labs
Steele Memorial Hospital (ID)
Stirling Royal Infirmary (Scotland)
Stony Brook University Hospital (NY)
Stormont-Vail Regional Medical Center (KS)
Stratford General Hospital (Canada)
Sunnybrook & Women's College Health Sciences Centre (Toronto, Ontario)
Sunnybrook Health Science Center (ON, Canada)
Swedish Medical Center (CO)
Sydney South West Pathology Service (Australia)
Taipei Veterans General Hospital (Taiwan)
Taiwan Society of Laboratory Medicine
Tampa General Hospital (FL)
Temple Univ. Hospital - Parkinson Pav. (PA)
Texas Department of State Health Services (TX)
The Bermuda Hospitals (Bermuda)
The Community Hospital (OH)
The Hospital for Sick Children (Canada)
The Nebraska Medical Center (NB)
The New York Hospital Medical Center of Queens (NY)
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Thomason Hospital (TX)
Timmins and District Hospital (Canada)
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TPMG Inc. (CA)
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Tripler Army Medical Center (HI)
Tuen Mun Hospital (Hong Kong)
Tufts New England Medical Center Hospital (MA)
Tuttle Army Health Clinic (GA)
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UCLA Medical Center (CA)
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